THE ROLE OF ALUMINUM IN PARENTERAL NUTRITION ASSOCIATED CHOLESTASIS IN INFANTS AND PIGLETS

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ABSTRACT

Aluminum is a known contaminant of parenteral nutrition (PN) solutions and it has been suspected to play a role in the development of parenteral nutrition associated cholestasis (PNAC). The primary purpose of my research was to monitor the relationship between serum aluminum level and the development of PNAC in the infants with gastrointestinal failure who required PN therapy. The secondary purpose was to develop a neonatal piglet model to compare different doses of aluminum or PN therapy with known aluminum level was associated with the development of PNAC.

Sixteen infants with gastrointestinal pathology were enrolled in the study. Serum aluminum and bilirubin (direct and indirect) concentrations were determined on day 0, 7, 14, and 21 of PN therapy. Five of sixteen (31.3%) infants developed PNAC by day 21. Serum aluminum levels in infants receiving PN peaked at day 7 of therapy and declined thereafter. There was no direct correlation between serum direct bilirubin and serum aluminum levels.

Twenty-four piglets, 2 to 4 days old, were placed into four groups: Control group (n=5); Low Al (aluminum) group (n=7), intravenous (iv) injection with aluminum dose at 20 $\mu g \cdot kg^{-1} \cdot day^{-1}$; High Al (aluminum) group (n=6), iv with aluminum dose at 1500 $\mu g \cdot kg^{-1} \cdot day^{-1}$; PN (parenteral nutrition) group (n=6), PN solutions with a mean aluminum intake at 37.8±14.3 $\mu g \cdot kg^{-1} \cdot day^{-1}$. The experiment period was 21 days. Serum bilirubin was significantly ($p<0.05$) elevated in the High Al and PN groups. Liver aluminum concentration was significantly ($p<0.05$)

elevated in all the experimental groups and the relationship was dose dependant. Serum, and urine concentrations of aluminum were significantly ($p<0.05$) elevated in High Al but not the Low Al and PN groups. Serum aluminum concentration was not correlated with serum total bilirubin levels.

Cholestasis developed by 21 days in five infants and in the piglets of two experimental groups (the High Al group and the PN group). High dose injection of aluminum may play a role in the development of PNAC in the neonatal piglets. The impact of aluminum may depend on the amount of pareternal aluminum intake and the presence of other potential factors such as lack of enternal feeding and individual physiological abilities.

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Acetyl Co A	acetyl coenzyme A
Al	aluminum
ALP	alkaline phosphatase
AMP	adenosine monophosphate
ASCN/ASPEN	American Society of Clinical Nutrition / American Society of Enteral Nutrition and Parenteral Nutrition joint commission
ATPase	adenosine triphosphatase
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CFTR	cystic fibrosis transmembrane regulator
cMOAT	cannalicular multispecific organic anion transporter
et al.	*et alia*; and others
FDA	Food and Drug Administration
g	gram(s)
GGT	gamma glutaryl transferase
ICP-AES	inductively coupled plasma atomic emission spectrometry
im	intramuscular
IU	International Unit
iv	intravenous
K^+	potassium ion
kg	kilogram(s)
MDR	multidrug resistant
mmol/L	millimole(s) per liter
MRP	multidrug resistance-associated protein
mg	milligram(s)
mL	milliliter(s)
Na^+	sodium ion
ND	not determined
NICU	neonatal intensive care unit
NRC	National Research Council
NTCP	sodium dependent taurocholate cotransporting polypeptide
OATP	organic anion transporting polypeptide
p	p-value or probability
PN	parenteral nutrition
PNAC	parenteral nutrition-associated cholestasis
r^2	correlation coefficient
RUH	Royal University Hospital
SD	standard deviation
SER	smooth endoplasmic reticulum
SGOT	serum glutamate oxaloacetate transaminase
SGPT	serum glutamate pyruvate transaminase

spgp	sister p-glycoprotein
SRC	Saskatchewan Research Council
TPN	total parenteral nutrition
UDCA	ursodeoxycholic acid
UDPGT	uridinediphosphoglucuronate glucuronosyl transferase
U/L	unit(s) per liter
µg	microgram(s)
µmol/L	micromole(s) per liter
vs	versus
%	percent or of each hundred
±	plus or minus

1. INTRODUCTION

1.1 Rationale

Parenternal nutrition (PN) is a vital tool in the supportive care of premature and critically ill infants. Cholestasis is a form of liver injury defined by a decrease or absence of bile flow into the duodenum (Erlinger, 1999). It is one of the most common complications of PN, especially in infants. The incidence of parenteral nutrition associated cholestasis (PNAC) was reported from 7.4% to 84% depending on birth weight, gestational age, as well as gastrointestional surgery, absence of oral intake, and diagnosis standards (Beath et al., 1996; Teitelbaum et al., 1997; Moss et al., 1999; Kubota et al., 2000). The disease can progress from mild cholestatic liver disease to cirrhosis and hepatic failure (Shattuck and Klein, 2000). PNAC has been shown to occur within two weeks of initiation of PN (Aynsley-Green, 1983). The diagnosis of PNAC is usually made with the findings of increased serum alkaline phosphatase, direct bilirubin, and the exclusion of other causes of neonatal cholestasis (Gremse and Balistreri, 1989; Btaiche and Khalidi, 2002). The pathogenesis of PNAC is still unclear. Research data show that many factors including prematurity, low birth weight, lack of enteral feeding, sepsis, and toxicity of PN solutions have been implicated as possible contributing factors (Ginn-Pease et al., 1985; Beath et al., 1996; Shattuck and Klein, 2000). However, the contributions of these factors in the development of PNAC are speculative.

One constituent of PN solutions that has been implicated in PNAC is the contaminant aluminum. Aluminum is one of the most abundant elements of the earth. Aluminum can be ingested as a contaminant of medications, food and water. Less than 1% of ingested aluminum is absorbed through the gastrointestinal tract (Moreno et al., 1994). Once absorbed, it is bound to the plasma protein transferrin and, to a lesser extent, albumin, then transported via the portal vein to the liver (Trapp, 1983; Bertholf et al., 1984). Aluminum has been found to deposit in lysosomes of macrophages and in lipofuscin granules of hepatocytes. Moreover, this aluminum accumulation was associated with severe ultrastructure lesions in these cells (Galle et al., 1987). Aluminum has been implicated in the progress of several diseases, including cholestasis, osteopenic bone disease, microcytic anemia, Alzheimer's disease, as well as encephalopathy (Klein et al., 1995; Koo et al., 1992; Recknagel et al., 1994). Absorbed aluminum is primarily excreted via the kidneys. (Greger and Sutherland, 1997).

Aluminum contamination of PN solutions has been known for more than two decades (Klein, 2003). Small volume additives to PN, such as heparin, calcium, phosphate salts, and blood products like albumin are contaminated with aluminum (ASCN/ASPEN, 1991; Popinska et al., 1999). Research on aluminum in neonatal parenteral nutrition solutions showed that the level of contamination ranged from 4.16 to 7.26 $\mu mol \cdot L^{-1}$ representing an average intake of 16.7 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (Moreno et al., 1994). This level significantly exceeds the 2 $\mu g \cdot kg^{-1} \cdot day^{-1}$ safe level that is recommended for the adult by the American Society of Clinical Nutrition / American

Society of Enteral Nutrition and Parenteral Nutrition joint commission (ASCN/ASPEN, 1991).

A few studies have investigated the role of aluminum in PNAC. Klein et al. (1984) examined hepatic aluminum accumulation in five children who developed PNAC while on long-term parenteral nutrition. All five demonstrated moderate to severe cholestatic changes in the liver. The hepatic aluminum concentrations were elevated, ranging from 5 to 27 times more than normal. In addition, significantly increased serum and urine aluminum levels were also found in these children. They concluded that aluminum loading might contribute to the pathogenesis of parenteral nutrition induced hepatocellular dysfunction (Klein et al., 1984). In a prospective study of 35 newborn infants, PN solutions accounted for 88.7% of the total aluminum intake (Moreno et al., 1994). Serum and urine aluminum levels in infants who required PN were significantly higher compared to a control group of formula fed infants. The PN group had greater hepatic tissue aluminum level than the controls. Klein et al. (1987) also used a piglet model to study the association between parenteral aluminum and PNAC. Increased serum bile acids and alkaline phosphatase levels were shown in the aluminum loaded piglets, suggesting that cholestasis had developed (Klein et al., 1987). The same research group also explored the relationships to dosage and duration of exposure in aluminum associated hepatobiliary dysfunction in rats (Klein et al., 1988). Further there was another report in which the intraperitoneal administration of aluminum produced portal inflammation in rats and a dose related elevation in the levels of serum bile

acids was found (Dermican et al., 1998). Both results suggested that aluminum exposure in rats associated with elevated serum bile acid concentration and diminished bile flow may play a role in PNAC (Klein et al., 1988; Dermican et al., 1998; Gonzalez et al., 2004).

Although previous studies suggested an association between aluminum and PNAC, the role of aluminum in PNAC is still unclear. The sample sizes of most human studies were small (n=5 in Klein's study and n=18 in Sedman's study) (Klein et al., 1984; Sedman et al., 1985). The experimental models used to inspect the character of aluminum in PNAC tended to be in older animals (Klein et al., 1987; Dermican et al., 1998) and with aluminum doses much larger than the contamination levels found in the hospitals today (Klein et al., 1987; Dermican et al., 1998; Gonzalez et al., 2004). Considering significant aluminum contamination exists, and the high risk of PNAC in infants, the proposed relationship requires further study.

1.2 Objectives

This study hypothesizes that aluminum is a contributing factor and plays an important role in PNAC in infants. The main objectives of the study were:

1. To investigate the role of aluminum in human infants who required PN.
    a. To monitor the relationship between serum aluminum and the development of PNAC.
2. To set up an animal model to clarify the role of aluminum in PNAC by using different doses of intravenous aluminum or PN solutions. This model will be a

neonatal model. The doses of parenteral aluminum used in this model will be similar to the dose found in the infants who are on PN therapy.

a. Develop a piglet model to study the relationship of aluminum and PNAC
b. To compare different doses of aluminum in the development of cholestasis
c. To determine if PN therapy with a known aluminum level is associated with the development of PNAC

To achieve these, there were two parts of the research: one was a human infant observational prospective study, and the other was an experimental piglet model study.

2. LITERATURE REVIEW

2.1 Neonatal liver structure, functions and bile flow

The liver is the largest solid organ in the body, constituting about 2% to 5% of the total body weight in adult and 5% in infant (Guyton, 2000). The liver is provided with a dual blood supply of portal blood derived from the intestinal (splanchnic) circulation, and arterial blood (Guyton, 2000). The basic functional unit of the liver is the lobule, a cylindrical structure, which is constructed around a central vein that empties into the hepatic veins and then into the vena cava. The lobule itself is composed principally of many hepatic cellular plates. Each hepatic plate is usually two cells thick, and between the adjacent cells are the small bile canaliculi that empty into bile ducts (Guyton, 2000). The hepatic sinusoids lie between the hepatic plates. The unique architectural arrangement of the intrahepatic vasculature into sinusoids interposed between hepatocytes greatly facilitates the exchange of material between blood and hepatocytes. There is a set of cells including sinusoidal endothelial cells, Kupffer cells, hepatic cells, and natural killer cell (also known as Pit cells), which line the sinusoids (Guyton, 2000). The main structural characteristic of the sinusoidal endothelial cell lining is the occurrence of fenestrae, which controls the interchange between blood and perisinusoidal space. Beneath this lining, lying between the endothelial cells and the hepatic cells is a narrow tissue space called the space of Disse. This space

connects with lymphatic vessels in the interlobular septa and places fluid in direct contact with hepatocytes (Guyton, 2000).

According to its structural organization, the liver has remarkable diverse functions, including (1) filitration and storage of blood; (2) metabolism of macronutrients, hormones and foreign chemicals; (3) formation of bile; (4) storage of vitamins and iron; and (5) formation of coagulation factors (Guyton, 2000). The liver serves as the primary regulatory site for energy metabolism, taking up and processing ingested nutrients for controlled distribution to extrahepatic tissues. In carbohydrate metabolism, the liver can store large amounts of glycogen, saving glucose while glucose in the blood is in excess. Gluconeogenesis in the liver occurs when the glucose concentration falls below normal. Therefore, the liver is especially important for maintaining a normal blood glucose concentration. In infants, the gluconeogenesis function of the liver is not fully developed. As a result, the blood glucose level of the unfed infant can fall to about 1.7 to 2.2 $mmol \cdot L^{-1}$, and the infant must depend on its stored fats for energy. The conversion of galactose and fructose to glucose also occurs in the liver. In addition, the liver synthesizes many important chemical compounds from intermediate products of carbohydrate metabolism. The liver is responsible for a major part of lipid metabolism: by the oxidation of fatty acids to supply energy. The liver also synthesizes large quantities of cholesterol, phospholipids, and most lipoproteins. About 80% of cholesterol is converted into bile salts. The remainder is transported in lipoproteins. Both cholesterol and phospholipids are used by cells to form membranes, intracellular

structures, and multiple chemical substances that are important to cellular function. Almost all the lipid synthesis from carbohydrates and proteins occurs in the liver as well, and fat is then transported in lipoproteins to adipose tissue to be stored. The liver is involved in protein metabolism: through the deamination of amino acids for gluconcogenesis, formation of urea for removal of ammonia from the body fluids, formation of plasma proteins, interconversions of the various amino acids, and synthesis of other compounds from amino acids (Guyton, 2000). About 90% of plasma proteins such as albumin, transferrin, and prealbumin, are formed by the hepatic cells. These proteins serve as transport proteins of drugs, nutrients, and hormones in addition to developing and maintaining plasma osmotic pressure. In the inflammatory response, the liver synthesizes many acute phase proteins such as C-reactive protein, ceruloplasmin and the complement factors (Chwals, 1995). However, the liver of the infant is immature, so the plasma protein concentration falls during the first weeks of life to 15 to 20% less than that for older children (Guyton, 2000). The liver also has the ability to synthesize the essential amino acids by transamination. Furthermore, the liver has a particular propensity for storing vitamins, especially vitamins A and D. Iron is stored as ferritin in the liver. A large proportion of the clotting factors used in coagulation are formed in the liver. The active chemical medium of the liver is well known for its capacity to detoxify or excrete into the bile many drugs, hormones and other substances.

Bile secretion is a major function of the liver cell. It is essential for excretion of endo- and xenobiotics, elimination of bilirubin and cholesterol, as well

as digestion and absorption of lipids and fat-soluble vitamins in the intestinal lumen (Scharschmidt, 2003). Approximately 0.5 grams per day of bile salts are formed by the hepatocytes. The primary constituents of bile are water, inorganic electrolytes, bile acids, cholesterol, phospholipids, and bilirubin (Gutyon, 2000). Three components contribute to the bile flow: 1.bile acid-dependent bile formation; 2. bile acid-independent canalicular secretion; 3. bile acid-independent flow of ductal origin.

Bile acid-dependent formation includes bile acid uptake, intracellular transport, and canalicular secretion by the hepatocyte (Kullak-Ublick et al., 2000). Bile acid uptake is mediated by two transporters: one is a sodium-dependent transporter, Na^+-taurocholate cotransporting polypeptide (NTCP); the other one is a sodium-independent transporter, organic anion transporting polypeptide (OATP) (Hagenbuch et al., 1991; Jacquemin et al., 1994; Kullak-Ublick et al., 1995). The energy for the NTCP is the transmembrane sodium gradient maintained by Na^+, K^+-ATPase on the basolateral membrane of the hepatocyte. Canalicular secretion is medicated by an ATP-dependent transport system and a voltage-dependent carrier (Nishida et al., 1991; Muller and Jansen, 1997; Trauner et al., 1998). The protein mediating ATP-dependent transport has been recognized as sister p-glycoprotein (spgp) (Gerloff et al., 1997; Trauner et al., 1998). Active secretion of bile acids by hepatocytes provides an osmotic drive for canalicular water and electrolytes. Therefore, bile flow is formed by an osmotic gradient created within the lumen of the bile canaliculus between adjacent hepatocytes. Bile acid is the most important

osmotically active solute. It generates the bile acid-dependent flow, which accounts for about 50% of total bile flow in all species (Boyer et al., 1992). Phospholipids are one part of bile acid-dependent flow and are transported by a protein of the multi-drug resistance (MDR) family: MDR3 (mdr2 in rodents). This P-glycoprotein translocates phosphatidylcholine from inner to the outer leaflet of the canalicular membrane (Oude Elferink et al., 1995; Frijters et al., 1997).

Canalicular excretion of glutathione and bicarbonate constitute the major components of the bile acid-independent fraction of the bile flow. The canalicular multispecific organic anion transporter (cMOAT) participates in the transport of glutathione and glutathione conjugates into the canalicular lumen (Oude Elferink et al., 1995). cMOAT is also referred to as multi-drug resistance-associated protein 2 (MRP2) (Oude Elferink et al., 1996; Trauner, 1997). Bilirubin is taken up by the hepatocytes by several carriers, including OATP. After conjugation, the bilirubin is secreted into the canalicular lumen by cMOAT or MRP2 (Keppler et al., 1997; Trauner et al., 1998).

Ductular bile flow is a bicarbonate-rich solution and is stimulated by secretin. Secretin receptors on the basolateral membrane of cholangiocytes bind secretin and activate cAMP production. Increasing cAMP stimulates cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel on the apical membrane of cholangiocytes, to secrete chloride into the biliary lumen (Trauner et al., 1998). Chloride then activates a chloride/bicarbonate exchanger to increase bicarbonate secretion. This exchanger is also located on the apical membrane.

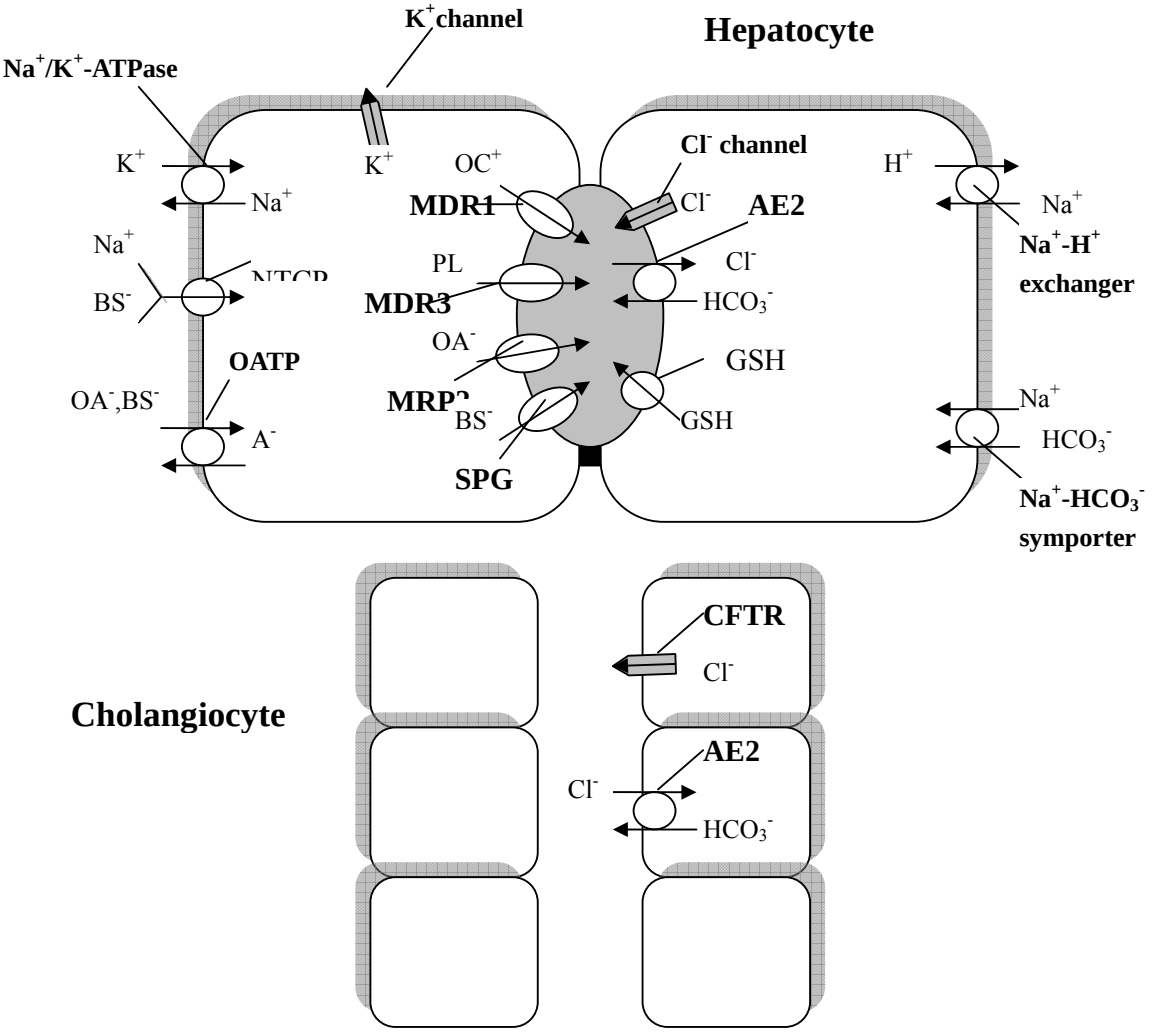
Bile is modified by the bile ductules and ducts, and delivered to the intestine and/or gallbladder (Boyer et al., 1992, Trauner et al., 1998). There is a linear relationship between bile flow and bile acid synthesis by the hepatocyte. This production is in turn regulated by the return of bile salts to the liver through the enterohepatic circulation (The liver secretes primary and secondary bile salts into the duodenum. Along the proximal and distal ileum, primary bile salts are reabsorbed into the portal circulation. This is called enterohepatic circulation) (Rees, 2002). In infants of any gestational age, the relative size of total bile acid pool is reduced and multiple steps of the enterohepatic circulation are less efficient compared with those of the normal adult (Hofmann, 1995).

Important advances have been made in recent years in the understanding of transport processes involved in bile formation as a result of cloning and characterization of several important transport proteins that mediate uptake, intracellular transport and secretion of organic compounds within the enterohepatic circulation (Muller and Jansen, 1997; Trauner et al., 1998; Hoffmaster et al., 2004) (Figure 2.1). These transport systems on both basolateral and canulicular membranes are involved in the mechanism of bile flow (Table 2.1). Any alteration of these transporters may cause an abnormality of bile flow (Lecureur et al., 2000).

Table 2.1 Function of transport proteins in bile formation
(Adapted from Muller and Jansen, 1997)

Transporter	Location	Function
	Bile acid-dependent bile flow	
NTCP	Basolateral membrane	Hepatic uptake of bile acids
Na^+-K^+ ATPase	Basolateral membrane	Removal of 2 Na^+ in exchange for 3 K^+, thus creating a favorable Na^+ gradient
cBAT (spgp)	Canalicular membrane	ATP-dependent canalicular transport of bile acid
MDR3	Canalicular membrane	ATP-dependent canalicular transport of phosphatidylcholine
	Bile acid-independent bile flow	
OATP	Basolateral membrane	Hepatic uptake of organic anions, neutral steroids, and type II organic cations
Na^+-K^+ ATPase	Basolateral membrane	See above, creates a favorable electro-chemical gradient for GSH transport
MRP2	Canalicular membrane	ATP-dependent canalicular transport of GSH-, glucuronide- and sulfate-conjugates and perhaps GSH
	Ductular secretion	
Cl^-/HCO_3^- exchanger	Apical membrane	HCO_3^- secretion in exchange for Cl^-
Cl^- channels e.g., CFTR	Apical membrane	Cl^- secretion

NTCP, human Na^+-taurocholate cotransporter; OATP, human organic anion transporting polypeptide; CFTR, cystic fibrosis transmembrane conductance regulator; cBAT, canalicular bile acid transporter; MDR3, multidrug resistance (MDR) protein 3; MRP1, human MDR protein; MRP2, human MRP1 homologue; GSH, glutathione.



NTCP: sodium taurocholate transporting polypeptide
OATP: organic anion transporting polypeptide
MDR1: multidrug-resistance-1 P-glycoprotein
MDR3: phospholipid transporter multidrug-resistance-3 P-glycoprotein
MRP2 or **cMOAT**: canalicular multispecific-organic-anion transporter
SPGP or **BSEP**: canalicular bile-salt-export pump
AE2: chloride-bicarbonate anion exchanger isoform 2
CFTR: cystic fibrosis transmembrane regulator
PL: phospholipid
OA^-: organic anion
BS^-: bile salt
OC^+: organic cation
A^-: anion

Figure 2.1 Transport system in the hepatocyte and cholangiocyte (Modified from Trauner et al., 1998)

2.2 Bilirubin metabolism in infants

Bilirubin is the degradation product of heme from red cells, as well as from muscle myoglobin and certain liver enzymes such as cytochromes and catalases. The first step in the breakdown of heme is catalyzed by heme oxygenase, an enzyme, which is found in the reticuloendothelial system, and in tissue macrophages as well as gut mucosa. The following step, reduction at the central C-10 carbon of biliverdin IXα to form bilirubin is catalyzed by biliverdin reductase in the cytosol (Tenhunen et al., 1969). Unconjugated bilirubin is transported in plasma bound to albumin. The binding of bilirubin to albumin increases with postnatal age, but it is reduced in sick infants and the presence of exogenous or endogenous binding competitors (Robertson et al., 1981). When the bilirubin-albumin complex passes through the hepatic circulation, bilirubin but not albumin is transported into the liver cell. There is an albumin receptor on the hepatocyte surface, and several transport proteins in the sinusoidal liver cell plasma membrane may be mediated in the transportation of bilirubin into the hepatocyte (Wolkoff et al., 1979). The principal bilirubin intracellular binding protein is ligandin, a glutathione S-transferase (Wolkoff et al., 1978). In monkeys, ligandin concentrations in the liver are low at birth but appear to reach adult values within 1-2 weeks (Levi et al., 1970). The bound bilirubin is then transferred to the smooth endoplasmic reticulum (SER) where conjugation takes place. The conjugation is catalyzed by bilirubin uridinediphosphoglucuronate glucuronosyl transferase (UDPGT), forming bilirubin monoglucuronide and diglucuronide. The role of this process is to convert the

water-insoluble unconjugated bilirubin to a water-soluble form, which can be excreted in the bile. The activity of UDPGT at birth is only 1% of adult values (Kawade and Onishi, 1981). Excretion of conjugated bilirubin into bile is an energy requiring process against the concentration gradient. When these conjugates are in the bile, they are subsequently transformed to urobilinoids through bacterial action in the gut lumen. Conjugated bilirubin may also undergo de-conjugation through the action of a β-glucuronidase, and the unconjugated bilirubin can then be reabsorbed through the intestinal mucosa back to the enterohepatic circulation. Alternate metabolic pathways for bilirubin may exist but are not currently known in human infants (Hansen, 2000). A schematic illustration of bilirubin metabolism is shown in Figure 2.2.

Almost every newborn infant will develop a serum unconjugated bilirubin >30 $\mu mol \cdot L^{-1}$ ($1.8 mg \cdot dL^{-1}$) during the first week of life. In general, the bilirubin level peaks between the 3rd and 5th days of life and declines thereafter (Maisels, 1988). In the infant the daily production of bilirubin is estimated to be about 8.5 $mg \cdot kg^{-1}$, which is more than double the production rate in adults (Maisels, 1988). The deficiency of ligandin in the infants may decrease the ability to retain bilirubin inside the hepatocyte and consequently may cause bilirubin to reflux into the circulation. However, there is no evidence that it is implicated in pathological jaundice (Hansen, 2000). The activities of UDPGT are low at birth and increase in early life to reach adult values at 14 weeks of age (Kawade and Onishi, 1981).

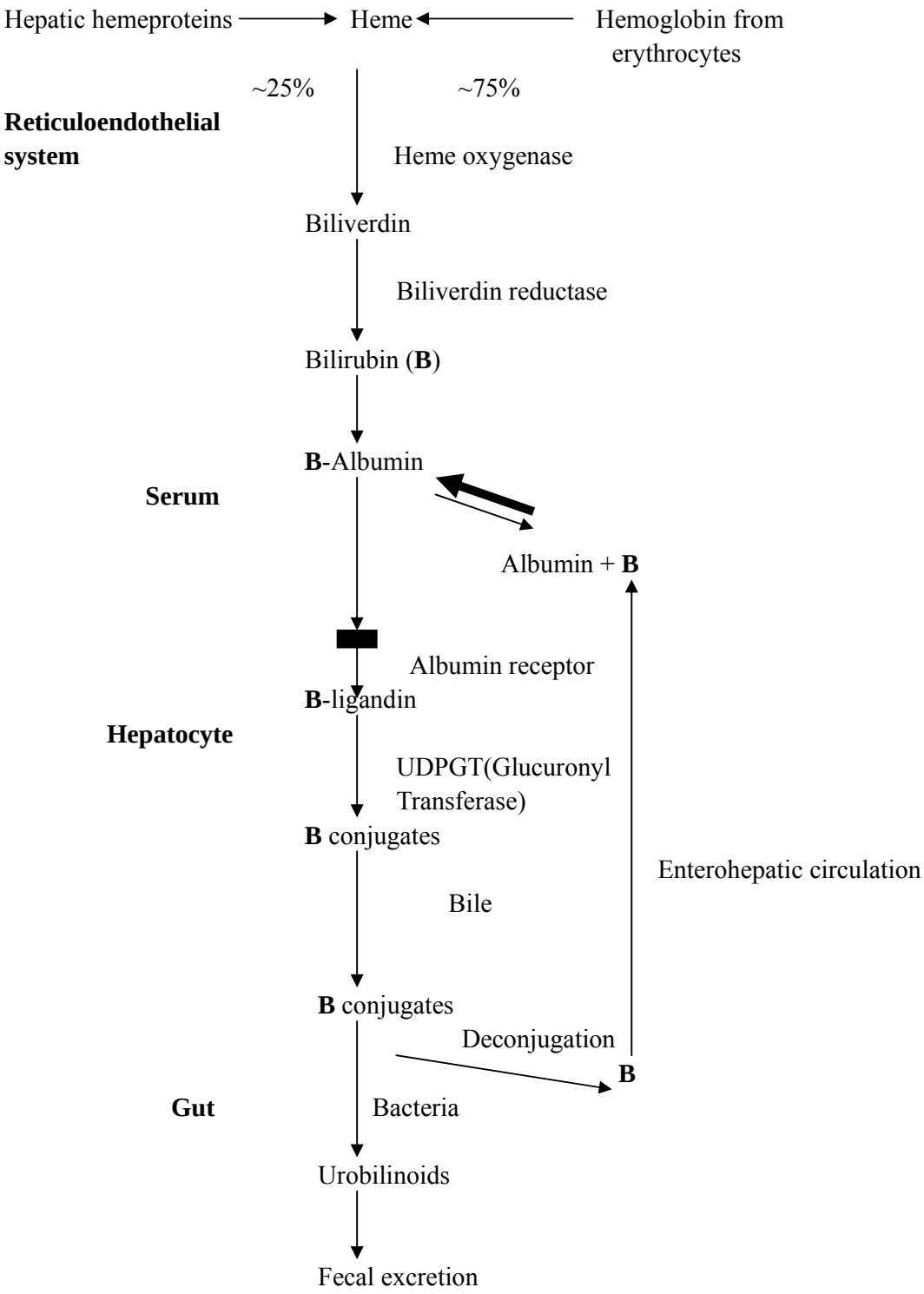


Figure 2.2 Bilirubin Metabolism

(Modified from Scharschmidt, 1982; Hansen, 2000)

In the first 3-4 days of life, conjugation is the rate-limiting step in bilirubin metabolism. Then the following next few days hepatic uptake and excretion are limiting, after that, excretion is the rate-limiting step during the rest of life (Hansen, 2000). Fluid and nutrient intake is limited in the newborn until breast milk production is established. As a result, there is a lack of binding vehicle in the intestinal lumen to transport bilirubin conjugates through the gut. The infant is born with a sterile gut, and the lack of the intestinal microbial flora decreases further breakdown of bilirubin and fecal urobilinoids excretion. In infants who are kept off oral feeds for reasons of severe illness, and in infants receiving inadequate nutrition, enterohepatic circulation is enhanced. It is possible that increased enterohepatic circulation is involved in the mechanism of neonatal jaundice (Hansen, 2000). Physiologic jaundice occurres in infants due to the increased breakdown of red blood cells in the presence of a low capacity for uptake, conjugation and excretion of bilirubin in the liver, as well as reabsorption of bilirubin from the gut because of the relatively low nutrient intake and thus reduced intestinal transit in the first few days of life (Hansen, 2000). However, the distinction between physiologic and pathologic jaundice in infants is often not clear. Each of the normally occurring mechanisms that produce hyperbilirubinemia may be exaggerated by both endogenous and exogenous factors.

2.3 Neonatal cholestasis

2.3.1 Pathophysiology of cholestasis

Cholestasis is a form of liver injury and is defined by a decrease or absence of

bile flow into the duodenum (Erlinger, 1999). The retention of bile in the liver causes hepatocyte injury. Morphologically, bile pigment can usually be found in hepatocytes, canaliculi, and sometimes Kupffer cells (Erlinger, 1999). Cholestasis may be caused by obstruction of the extrahepatic bile ducts, obstruction of the intrahepatic bile ducts, or alteration of the patterns of the secretion of bile by the hepatocytes (Table 2.2) (Erlinger, 1999). Up to 50% of the neonatal cholestasis was due to genetic/metabolic diseases or parenteral nutrition (Stormon et al., 2001).

Extrahepatic cholestasis

The main reason for extrahepatic cholestasis is obstruction of extrahepatic bile ducts. Dilatation of bile canaliculi with a loss of microvilli and alterations of intercellular junctions can be found in the bile duct ligation cholestasis model. These alterations increase canlicular permeability and allow a "regurgitation" of bile constituents into hepatic lymph and blood (Accatino et al., 1981). Bile duct ligation also induces modifications in hepatocytes. Various enzymes and carriers of the hepatocyte plasma membrane are redistributed to the sinusoidal and lateral pole of the cell. The modified polarity of the membrane causes the substrates to re-secrete into blood after their entry into the hepatocyte. Because of the change in hepatocyte metabolism, alkaline phosphatases and cholesterol production increase. Jaundice is severe in this kind of mechanical biliary obstruction. Bile pigment may be seen in the lumina of cholangioles or acinar bile ducts (Erlinger, 1999). When biliary obstruction is prolonged, it leads to bile ductular proliferation, fibrosis, and secondary biliary cirrhosis (Erlinger, 1999). The specific mechanisms of ductular

Table 2.2 Common causes of cholestasis (Adapted from Erlinger, 1999)

1. **Causes of obstruction of extrahepatic bile ducts**
   - Common bile duct stone
   - Pancreatic carcinoma, chronic pancreatitis, pancreatic cyst
   - Carcinoma of the ampulla of Vater
   - Bile duct strictures
   - Parasites
   - Primary sclerosing cholangitis
   - AIDS cholangiopathy
   - Portal vein thrombosis
   - Hodgkin's disease, lymphoma
   - Choledochal cyst
   - Biliary atresia
2. **Causes of obstruction of intrahepatic bile ducts**
   - Primary biliary cirrhosis
   - Space-occupying lesions
     - Primary or secondary liver cancer
     - Lymphoma
     - Amyloidosis
     - Vanishing bile duct syndrome
     - Allograft rejection
     - Graft-versus-host disease
     - Alagille's syndrome
     - Idiopathic adult ductopenia
     - Primary sclerosing cholangitis
     - Hodgkin's disease
     - Cystic fibrosis
3. **Causes of hepatocellular cholestasis**
   - Genetic
     - α_1-antitrypsin deficiency
     - Byler syndrome
     - Abnormal bile acid synthesis
     - Porphyria
     - Cholestasis of pregnancy
     - Benign recurrent intrahepatic cholestasis
   - Acquired
     - Pure cholestasis without hepatitis: drugs (i.e., estrogen)
     - Cholestatic hepatitis: virus, drugs, alcohol
     - Bacterial infections
     - Total parenteral nutrition
     - Paraneoplastic cholestasis: renal carcinoma, Hodgkin's disease
     - Miscellaneous

proliferation and fibrosis are not clear. In animals, the changes observed after biliary obstruction are reversible when obstruction is relieved and adequate bile flow is restored (Zimmermann et al., 1992).

Intrahepatic cholestasis

Intrahepatic cholestasis may result from obstruction of intrahepatic bile ducts or from alteration of bile secretion by the hepatocytes (hepatocellular cholestasis). For obstruction of intrahepatic bile ducts, the mechanisms are basically the same as the exrahepatic obstruction with the exception of cystic fibrosis. The function of cystic fibrosis transmembrane conductance regulator (CFTR) is impaired with decreased chloride and bicarbonate secretion (Roy et al., 1982). This leads to a decrease in ductal bile flow. The decreased bile flow results in mucous precipitation and obstruction of small bile ducts. The consequence of obstruction is ductular proliferation, inflammation, focal fibrosis, and focal biliary cirrhosis (Roy et al., 1982). Regarding hepatocellular cholestasis, the impairment of bile secretion by the hepatocytes may be due to hemodynamic disturbances or alteration of the sinusoidal, intracellular, or canalicular steps of the hepatic transport of bile (Eringler, 1999). A foamy transformation of the cytoplasm of hepatocytes, Kupffer cells, and biliary epithelial cells can be seen in the prolonged cholestasis. Many disorders, including alcoholic liver disease, drug-induced hepatotoxicity, viral hepatitis, and various developmental and metabolic diseases, can be associated with intrahepatic cholestasis (Eringler, 1999).

In infants, several factors contribute to decrease bile flow including

inefficient bile acid intake, impaired bile acid conjugation, qualitative and quantitative deficiencies of bile acid synthesis, and decreased hepatocellular excretion (Table 2.3). Immaturity of hepatic function creates the potential for further decreases in bile flow in response to any insult in infants, such as endotoxin or parenteral nutrition (Gremse and Balistreri, 1989). In addition, other conditions including prematurity, hypoxia, hypoperfusion, sepsis, hepatitis, and fasting, may further impair hepatic excretory function (Btaiche and Khalidi, 2002).

2.3.2 Molecular mechanisms of cholestasis

In general, cholestasis results from defects in the regulation and expression of the membrane transporters that generate bile secretion and from cellular events that impairs signal transduction pathways, cytoskeletal structures, tight and gap junctions, and the targeting of intracellular vesicles (Shaffer, 2002; Hyogo et al., 2001). Results of recent molecular studies indicate that decreased expression of transport systems contributes significantly to the impairment of transport functions that result in or maintain cholestasis (Crocenzi et al., 2003; Geier et al., 2005; Lee et al., 2000; Geier et al., 2003). Some of these defects are related to hereditary defects by which mutations or polymorphisms result in impairment of transporter protein expression (Table 2.4). In rats with obstructed bile ducts, expression of ntcp and oatp (NTCP and OATP in human), which are responsible for bile acid uptake, is reduced due to a decrease in transcription (Gartung et al., 1996; Dumont et al., 1997). The down-regulation could prevent the accumulation of potentially toxic bile acids and organic anions within the hepatocytes (Geier et al., 2005). The down-regulation of

Table 2.3 Manifestations of underdeveloped bile acid transport and metabolism in infants (Gremse and Balistreri, 1989)

Serum bile acid levels are increased
Hepatic uptake of bile acids is decreased
Bile acid efflux is enhanced
Lobular gradient is absent
Conjugation, sulfation, glucuronidation are decreased
Qualitative and quantitative defects in synthesis are present
Bile acid pool size is decreased
Bile acid secretion rate is decreased
Intraluminal concentrations of bile acid are low
Ileal active transport of bile acids is low

Table 2.4 Molecular changes of hepatocellular transport systems in humans (Scharschmidt, 2003; Elferink and Groen, 2002;Trauner et al., 1998)

Disease	Molecular Change
Hereditay:	
Progressive familial intrahepatic cholestasis(PFIC)	
PFIC-1(low serum γ-glutamyltransferase)	Mutation in P-type ATPase
PFIC-2(low serum γ-glutamyltransferase)	Mutation of BSEP gene
PFIC-3(high serum γ-glutamyltransferase)	Canalicular BSEP protein absent Canalicular MDR3 protein absent
Benign recurrent intrahepatic cholestasis(BRIC)	Mutation in P-type ATPase
Dubin-Johnson syndrome	Canalicular MRP2 protein absent
Acquired:	
Primary biliary cirrhosis	AE2 mRNA and protein reduced; hepatocytes and cholangiocytes are affected; MDR3 mRNA levels unchanged
Extrahepatic biliary atresia	NTCP mRNA reduced. Inverse correlation with serum bilirubin levels; increase after successful Kasai procedure. BSEP protein maintained at canalicular membrane
Primary sclerosing cholangitis	OATP mRNA increased, possibly diminishing hepatic retention of organic anions
Extrahepatic biliary obstruction	MDR1 and MDR3 mRNA increased. Direct correlation with serum bilirubin levels.

cmoat (mrp2) (MRP2 in human) could contribute to impair biliary excretion (Trauner, 1997). However, most of the alterations that occur in both hereditary and acquired cholestatic disorders are secondary responses rather than primary causative changes. The expression of specific membrane transporters adapts to a variety of forms of cholestatic liver injury in a manner that tends to diminish the hepatic uptake of bile salts from portal blood, while at the same time maintaining or in some instances up-regulating mechanisms that may help to facilitate the exit of bile salts and other toxic substances from hepatocytes. Thus, transporters on the basolateral sinusoidal membrane that normally function to selectively remove bile salts and other substances from portal blood are usually down- regulated during cholestasis by transcriptional or posttranscriptional mechanisms (Gartung et al., 1996; Dumont et al., 1997; Trauner et al., 1998; Lee et al., 2000). On the other hand, some canalicular transport proteins, particularly the MDR homologues, are either not impaired or may actually be up-regulated (Bhunchet and Fujieda, 1993; Tazuke et al., 2004).

Profound changes in the cytoskeleton of the hepatocytes, including disruption of microtubules, increases in intermediate filaments, and accumulation of disorganized bundles of actin microfilaments in the pericanalicular domain, are shown in most human and animal cholestatic liver disorders (Hyogo et al., 2001). These changes result in the loss of apical microvilli and diminished contractility of the canalicular membrane and may also contribute to the leakiness of tight junctions between cells (Trauner et al., 1998).

2.3.3 Parenteral nutrition associated cholestasis

2.3.3.1 Overview

Since the development of parenternal nutrition (PN) about three decades ago, many patients who are unable to tolerate food have survived and recovered. PN has become a vital tool in the supportive care of premature and critically ill infants. Although undoubtedly life-saving in many instances, PN is associated with several complications, including metabolic imbalance, recurrent thromboembolism, and liver dysfunction (Taylor et al., 1991; Kelly, 1998). Parenteral nutrition associated cholestasis (PNAC) is one of the most common complications in infants which accounts for appreciable morbidity and occasional mortality (Kelly, 1998). The incidence has been reported from 7.4% to 84% of the patients receiving PN (Beath et al., 1996; Teitelbaum et al., 2001). The wide variations in the frequency reported may be due to the nature of the patient population studied and many other confounding factors, such as gastrointestinal surgery, prematurity, the duration of PN, different diagnostic standards and the presence of sepsis. 25% of the gastrointestinal failure infants developed PNAC was found in our unpublished data (Arnold, 2004). A recent study in Japan found an incidence of 25% from 1992 to 1996 with an associated mortality of 3% in their neonatal wards (Kubota et al., 2000). Kelly's research in children on long-term PN shows that 40% to 60% of patients develop some degree of hepatic dysfunction (Kelly et al., 1998).

Clinically, a conjugated hyperbilirubinemia with elevations in levels of alkanine phosphatase (ALP) and serum aminotransferases may be present. Jaundice

and hepatomegaly are the most common physical findings. The diagnosis of PNAC is made after other causes of neonatal cholestasis have been excluded (Gremse and Balistreri, 1989). Histological changes are nonspecific and vary considerably among patients (Btaiche and Khalidi, 2002; Zambrano, et al., 2004). Giant cell transformation, hepatocellular damage, extramedullary hematopoiesis, portal fibrosis, changes characteristic of neonatal hepatitis may be observed. These changes can be noted within 10 days of PN introduction (Briones and Iber, 1995; Hodes et al., 1982; Dahms and Halpin, 1981).

Biochemical abnormalities may persist for months after jaundice is resolved. This places the infant at risk for chronic liver disease and its complications. Less than 10% of PNAC patients progressed to cirrhosis, portal hypertension and liver failure (Kubota et al., 2000). The risk factors for the progression are not well delineated. Because of the lack of understanding of the etiology of PNAC, the treatment of most patients is empiric and supportive. Available evidence suggested that discontinuation of PN and institution of enteral feeding were useful for resolving PNAC, but not all (Kelly, 1998; Hodes et al., 1982; Moss et al., 1993; Dermican et al., 1999;). Cycling of PN, ursodeoxycholic acid (UDCA) and phenobarbital or prophylactic antibiotics has been used with some success (Capron et al., 1993; Collier et al., 1994; Spagnuolo et al., 1996; Chen et al., 2004).

2.3.3.2 Potential factors in neonatal parenteral nutrition associated cholestasis

The pathogenesis of PNAC is still unclear. Recently most of the studies suggest that the causes of this disease in infants often are multifactorial (Drongowski

and Coran, 1989; Beath et al., 1996; Kubota et al., 2000; Btaiche and Khalidi, 2002). PNAC typically occurs in the clinical setting of multiple factors that may damage the liver. Premature birth, duration of PN, lack of enteral feeding, sepsis, imbalanced nutrients, potentially toxic drugs, and contamination of PN solutions may all contribute to PNAC (Figure 2.3). However, in individual patients, the relative contribution of each of the potential factors is highly variable (Merrit, 1986; Btaiche and Khalidi, 2002).

Beale et al. (1979) found the incidence of PNAC in a series of 62 infants was 23% overall, with an incidence of 50% in infants with birth weight under 1000g; 18% in those from 1000 to 1499 g; and 7% in those from 1500 to 2000 g. Several studies concur that PNAC is more common in extremely low birth weight or premature infants (Kelly, 1998). It has been suggested that the immaturity of the hepatobiliary system may play a role in PNAC. The relative reduced pool size of total bile acid, low specific enzyme activity, ineffecient hepatocyte uptake and excretion of bile acids, and immature enterohepatic circulation can decrease the bile flow (Hofman, 1995; Farrell and Balistreri, 1986). However, these immaturities also exist in infants of any gestational age. The agreement is the high risk of PNAC in infants because of these physical immaturities. In a retrospective study, 17 of 32 infants who received PN therapy at least 7 weeks developed cholestasis (Drongowski and Coran, 1989). There were significant differences between the cholestasis and noncholestasis infants in number of operations (13 vs 6, $p = 0.0407$), and days until enteral feedings were started (33.1 vs18.9, $p = 0.0289$). However, no difference

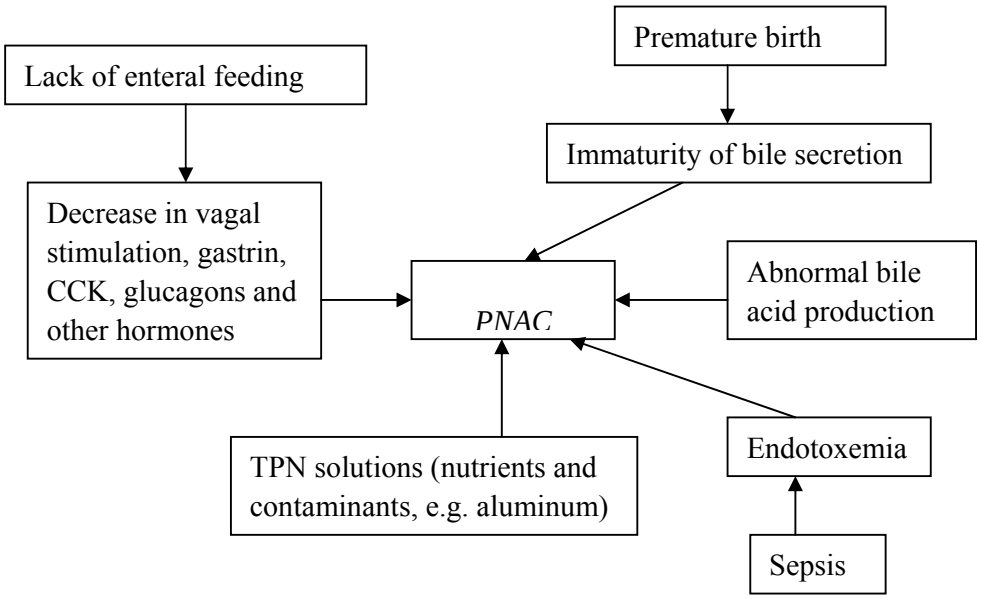


Figure 2.3 Potential factors of PNAC in infants (Modified from Gremse and Balistreri, 1989)

was found in birth weight, gestational age, days from birth to the start of PN, or respiratory distress in the study (Drongowski and Coran, 1989). The relationship between the premature birth and PNAC is still not well delineated. More studies need to be done to define the birth weight or gestation age and the contribution to PNAC.

Lack of enteral feeding is one feature of PN and recognized by some studies as a risk factor in PNAC (Drongowski and Coran, 1989; Quigley et al., 1993; Hofmann, 1995). The mechanism was thought that because of absent substrate, the normal pattern of interdigestive migrating motor complex is not maintained, and intestinal motility is slow. The secretion of the hormones cholecystokinin (CCK) and secretin is decreased. CCK is responsible for gallbladder contractility thus preventing biliary sludge formation (Spagnuolo et al., 1990). Moreover, fasting leads to atrophy of the enterocytes and decreased ileal absorption of the primary bile salt taurocholate (Matsumura et al., 1993). The decreased absorption of bile acids from the gut resulted in increased concentrations of cholestatic secondary bile acids which are toxic bile acids causing cholestasis (Foulin-Fortunet et al., 1982). Although researchers recognized the possible mechanism of lack of enternal feeding in PNAC, the results from further studies seem to be controversial. Increasing the CCK levels did not significantly reduce the incidence of PNAC (Aynsley-Green et al., 1983; Moss and Amii, 1999; Teitelbaum et al., 2005). Other researches in addition demonstrated that the use of enteral feeding didn't improve the symptom of progressive PNAC, especially intrahepatic cholestasis (Hodes et al., 1982; Moss et

al., 1993; Dermican et al., 1999). Two review studies also pointed out that enteral starvation did not correlate significantly with the development of PNAC in infants (Beath et al., 1996; Zambrano et al., 2004). At this point, the role of lack of enteral feeding in PNAC is still contentious.

Sepsis has been found to be associated with an increased risk of PNAC (Moss et al., 1996; Beath et al., 1996). The researchers found that episodes of sepsis were associated with a 30% increase in the bilirubin level. The septic events that happened in the PNAC infants were significantly higher than the non-cholestatic infants (Beath et al., 1996). In one series including infants on short-term PN, the incidence of cholestasis was 26% in those with infection, and it did not occur in those without infection (Wolf and Pohlandt, 1989). The theory of sepsis in PNAC is thought to be an endotoxin which is produced by gram-negative bacteria and causes the release of cytokine such as tumor necrosis factor-α and interleukin-I (Beath et al., 1996). The used of antibody against tumor necrosis factor-α improved the PNAC in the case report (Forrest et al., 2002). More clinical trials are needed to verify the treatment of PNAC. Animal studies have demonstrated that bacterial endotoxins cause inflammation and fibrosis in the liver. However, a pathology study in 24 infants showed that there was no significant relationship between sepsis and liver injury (Zambrano et al., 2004). The exact mechanism on which sepsis leads to cholestasis remains unclear.

The nutritional components of PN solutions are always prominent on the list of suspected contributors to PNAC. There has been significant research attempting

to define the role of the individually infused nutrient in the pathogenesis of cholestasis (Forchielli and Walker, 2003).

Investigators found that the incidence of cholestasis was directly related to the volume of amino acids infused by affecting the canalicular membrane of the hepatocyte (Black et al., 1981; Merritt, 1986; Kelly, 1998). In a prospective controlled study, infants with high amino acid infusion (3.6 $g \cdot kg^{-1} \cdot day^{-1}$) developed PNAC earlier (27 days, $p<0.01$) than the low amino acid infusion (2.3 $g \cdot kg^{-1} \cdot day^{-1}$) group and achieved a significantly greater peak direct bilrubin level (143 $\mu mol \cdot L^{-1}$ versus 54.4 $\mu mol \cdot L^{-1}$, $p<0.001$) (Vileisis et al., 1980). In rats, a high concentration of glycine, a sodium-dependent transport amino acid, inhibits the hepatic basolateral membrane uptake of the bile acid taurocholate (Bucuvalas et al., 1985). Alternatively, the deficiencies of specific amino acids, such as taurine, have been suggested as possible causes of PNAC (Dorvil et al., 1983). Taurine is an essential amino acid for infants due to a relative lack in the enzyme cystathionase for synthesis. Taurine is the primary amino acid used for conjugation of bile acids. The deficiency of taurine in PN solutions may contribute to the development of PNAC. However, there is little supporting evidence to indicate that the pediatric amino acid solutions which include taurine have reduced the incidence of PNAC in infants. Methyl donor amino acids, such as methionine or serine have been evaluated in cholestasis with mixed results. Belli et al. (1987) suggested that the additional methionine or serine may protect against the development of PNAC, whereas others found that methionine may be toxic to the liver (Coran et al., 1987;

Moss et al., 1993). Recently published studies also suggested that PNAC was associated with the different type of amino acid solution administered (Suita et al., 2002; Wright et al., 2003). However, which type of formulation may be less likely to cause PNAC needs further study.

Carbohydrate overload may overwhelm the hepatic glucose-oxidizing capacity and could cause hepatocellular enlargement and other hepatic injuries. The damages of liver cells are sufficient to compress the canaliculi and obstruct bile flow (Hirai et al., 1979). But in adults, carbohydrate overload produces steatosis not cholestasis. A study from Japan investigated the dextrose overload and the effects of amino acids in PNAC in newborn rabbits. PNAC was found in the high dose dextrose group (25 $g \cdot kg^{-1} \cdot day^{-1}$) (Hata et al., 1993). However, this same study also showed that PNAC was prevented by adding an appropriate volume of amino acids. Considering the PN solutions used in the hospitals now, carbohydrate overload is rare.

The lipid emulsions also have been investigated. The association between lipid emulsions and PNAC was reported in children on the long-term parenteral nutrition (Colomb et al., 2000). Conversely, other studies showed a protective effect of lipid emulsions on the liver when a balanced nutritional regimen was provided (Oshita et al., 2004; Reif et al., 1991). A recent study found that fish oil based lipid emulsions can reduce the incidence of PNAC compared with soybean-derived emulsions in neonatal piglets (Van-Aerde et al., 1999). It is thought that different fatty acid composition may modify bile composition due to the

changes of hepatocyte membrane composition and function. Plant sterol is another lipid component that has been investigated. Lipid hydroperoxides and phytosterols have been measured in high levels in lipid emulsions. Plant sterols may relate to the histologic evidence of peroxidation of cell membranes and have been shown to cause a reduction in bile secretion in experimental models (Wispe et al., 1985; Iyer et al., 1998). The lipid now used in the clinics is a mixed emulsion with soybean oil and egg phospholipids. Little evidence is available in human studies. In fact, cholestasis was reported in infants before the routine use of parenteral lipid emulsions in nutritional support (Farrell et al., 1986). This suggests that fat emulsions may contribute to PNAC but probably are not the major cause of PNAC.

Trace minerals including zinc, manganese, copper, chromium, selenium and iodine are added to PN solutions (ASCN/ASPEN, 1991). Because some of the trace minerals are excreted in bile, such as manganese and copper, patients with pre-existing hepatic dysfunction are at particular risk for toxicity (Reynolds et al., 1994; Ferenci et al., 2002). The accumulation of manganese and copper in the liver associated with high blood concentrations has been documented in adults and children on PN (Kelly, 1998; Shike et al., 1981; Fok et al., 2001). It is uncertain whether high serum levels lead to cholestasis or are the result of decreased mineral secretion in bile.

2.4 The toxicity of aluminum

There is evidence that the contaminants of PN solutions, such as aluminum, may play a role in PNAC. Details of aluminum in PNAC will be discussed in the

following section.

2.4.1 Metabolism of aluminum

Aluminum is the third most abundant element of the earth's crust. The intake of aluminum from natural sources with food and beverages accounts for 90-95% of the total daily intake in most adults. The absorption of aluminum via the gastrointestinal tract is generally less than 1% (Greger and Sutherland, 1997). Parenteral aluminum exposure usually occurs via contaminated PN solutions, some intravenous (iv) drugs, and blood products such as albumin.

After absorption, aluminum is bound in the plasma primarily to transferrin, and to a lesser extent to albumin, as well as in free form (Bertholf et al., 1984; Greger and Sutherland, 1997). Aluminum can then be delivered to the liver by transferrin (Greger and Sutherland, 1997; Klein et al., 1993). The accumulation of aluminum in lysosomes of macrophages and in lipofuscin granules as well as in the phagolysosomes of hepatocytes has been found under electron microscopy. These depositions of aluminum created severe ultrastructural lesions in the liver cells (Fiejka et al., 1996; Galle et al., 1987).

The major route of aluminum elimination is by urine and a small amount excreted in bile. Most blood aluminum is cleared in the urine in healthy adults (Greger and Sutherland, 1997). The excretion data in infants is insufficient. However, infants have immature renal function that may negatively effect aluminum excretion.

The toxicity of aluminum was first described in uremic patients undergoing

hemodialysis (Berlyne et al., 1970). Subsequently toxic effects due to high aluminum body loads were observed in several conditions. The risk of aluminum toxicity is dependent on the length of exposure, the dose and renal function. Aluminum toxicity has also been implicated in the pathogenesis of several diseases, including PNAC, Alzheimer's disease, Parkinson's disease, and osteomalacia (Klein et al., 1995; Koo, 1992; Savory et al., 1996).

2.4.2 Aluminum in parenteral solutions

Aluminum contamination of PN solutions was first identified in 1982, notably in the casein hydrolysate, which was the protein source at the time. Aluminum was found in urine, blood, and bones of patients receiving these solutions (Klein et al., 1982). The same research group also identified the aluminum level in the PN solutions delivered to the patients in the neonatal intensive care unit contained 327±130 $\mu g \cdot L^{-1}$, which would deliver approximately 30 $\mu g \cdot kg^{-1} \cdot day^{-1}$ to a preterm infant (Klein et al., 1991). ASCN-ASPEN Working Group recommended that aluminum intake below 2 $\mu g \cdot kg^{-1} \cdot day^{-1}$ was safe, 15-30 $\mu g \cdot kg^{-1} \cdot day^{-1}$ was unsafe and 60 $\mu g \cdot kg^{-1} \cdot day^{-1}$ was toxic for adults (ASCN/ASPEN, 1991). For infants, several studies suggested that the toxic level for parenteral aluminum intake was much lower that the adult level. Bishop et al. (1997) demonstrated that loads of 15-30 $\mu g \cdot kg^{-1} \cdot day^{-1}$ in PN solutions given to premature infants began to approximate toxic levels. The aluminum content in PN solutions ranged from 112 to 196 $\mu g \cdot L^{-1}$, representing a mean daily intake of 16.7 $\mu g \cdot kg^{-1} \cdot day^{-1}$ in neonatal patients (Moreno et al., 1994). A more recent study showed that the contamination levels of

aluminum in PN solutions were higher, particularly in calcium salts, phosphates, and trace elements (Popinska et al., 1999). Aluminum content was also found in heparin and blood products such as albumin (Recknagel et al., 1994; Davis et al., 1999). In 2000, the Food and Drugs Administration (FDA) of the United States of America published guidelines for aluminum contamination of PN solutions. Large volume solutions should not contain more than 25 $\mu g \cdot L^{-1}$ aluminum. For small volume solutions, the maximum level of aluminum must be stated on the label (FDA, 2000).

2.4.3 Aluminum and parenteral nutrition associated cholestasis

There was some evidence of hepatic aluminum accumulation in children and infants who received PN. In 1984, Klein et al. (1984) investigated 5 children, aged 18 to 34 months, who developed PNAC while on PN for 18 to 33 months. Liver enzymes (SGOT, SGPT, ALP), total or direct bilirubin were increased. They also found elevated serum, urine, and hepatic aluminum levels in these patients. Hepatic aluminum accumulation ranged from 5 to 27 times normal concentrations. Histopathology of liver biopsies from these patients demonstrated periportal fibrosis, bile duct proliferation, inflammatory cells, cellular necrosis and nonuniform nodular regeneration (Klein et al., 1984). Sedman et al. (1985) prospectively studied 18 premature infants on PN and compared with 8 term infants without intravenous therapy. They found premature infants who were on PN had high plasma and urinary aluminum concentrations as compared with normal controls. In 1994, Moreno et al. (1994) reported an observational study of 35 infants who required PN.

The average duration of PN was 15.6±8.7 days. Similar aluminum accumulation results were found in blood and urine samples. Aluminum accumulation in tissue was confirmed in 3 infants whom aluminum levels were studied in autopsy samples. In addition, they showed that PN solutions were the main source of aluminum representing 88.7% of total aluminum intake (Moreno et al., 1994). Based on the evidence of aluminum accumulation in infants who received PN, elevated liver enzymes and total/direct bilirubin, high serum and urine aluminum level, as well as histology findings in PNAC infants, researchers concluded that aluminum loading could contribute to the pathogenisis of PNAC (Klein, 2003).

Several animal models have been used to further study aluminum in PNAC. Klein et al. (1987) followed their human studies by using a piglet model to investigate the relationship of aluminum and PNAC. Eight piglets, 6 weeks old, were divided into experimental and control groups. The experimental group was given parenteral aluminum 1.5 $mg \cdot kg^{-1} \cdot day^{-1}$ while the control group received heparinized saline for 50 days. At the end of the study, serum total bile acid levels, mean alkaline phosphatase (ALP), and hepatic aluminum levels were significantly elevated in the experimental piglets, indicating that cholestasis may have developed. The deposit of aluminum in hepatocyte lysosomes of the experimental animals was also seen by electron microscopy (Klein et al., 1987). In 1988, the same research group studied different doses and duration of exposure of aluminum associated hepatobiliary dysfunction in rats. Three experimental groups received intravenous aluminum as follows: 1. 5 $mg \cdot kg^{-1} \cdot day^{-1}$ for 14 days; 2. 1 $mg \cdot kg^{-1} \cdot day^{-1}$ for 14 days;

3. 5 $mg \cdot kg^{-1} \cdot day^{-1}$ for 7 days. The control animals received saline. After the experiment period, bile and urine were collected for 3 hours. They found that hepatic aluminum levels and total serum bile acid concentration were significantly elevated in experimental rats in 14 days than in 7 days compared with the control group. Bile flow was reduced by 33% in 5 $mg \cdot kg^{-1} \cdot day^{-1}$ (7days and 14 days) groups but not in 1 $mg \cdot kg^{-1} \cdot day^{-1}$ (14 days) group. Additionally, an inverse correlation between hepatic aluminum and bile flow but not serum bile acid concentration was found. They concluded that aluminum exposure in rats may be associated with pathogenesis of parenteral nutrition-induced hepatobiliary dysfunction (Klein et al., 1988). Demircan et al. (1999) investigated the effects of administration of PN and aluminum in rats. Their experimental group received PN and/or aluminum. The level of aluminum in PN solutions and intravenous aluminum dose used in this study were equal to 66.85 $\mu g \cdot kg^{-1} \cdot day^{-1}$. The control group received intraperitoneal saline. The experiment lasted for 7 to 14 days. Portal inflammation and hepatic aluminum accumulation was found in all experimental subjects. A dose related elevation in the levels of serum bile acids was present (Demircan et al., 1999). A recent study demonstrated that a chronic aluminum exposure damaged the biliary secretory function in rats. The experimental group received aluminum intraperitoneally at 27 $mg \cdot kg^{-1}$, three times a week for 90 days. The results showed that cholestasis had developed. The ability of hepatic transport of organic anions was impaired by decreasing both sinusoidal uptake and canalicular excretion (Gonzalez et al., 2004). Duerksen et al. (1996)

studied PN-induced liver disease in the newborn piglet. For three weeks, eight piglets received PN and nine piglets were fed sow's milk. They found that liver and serum bilirubin were elevated in the PN group. Bile flow was significantly reduced and bile composition was altered in the PN group. They suggested that PNAC may be characterized by decreased bile acid secretion, elevated serum bilirubin and reduced bile flow (Duerksen et al., 1996). However, the contamination level of aluminum of PN solutions in this study was unknown. The following table is a summary of previous animal studies (Table 2.5).

Although previous reports suggested an association with aluminum and PNAC, the exact role of aluminum in PNAC is still unclear. The sample sizes of most human studies are small. The experimental models used to explore the role of aluminum in PNAC tended to be in older animals and with aluminum dose much larger than the contamination levels seen today. Considering that significant aluminum contamination exists, and the high risk of PNAC in infants, the proposed relationship requires further study.

2.5 Piglet as a model in human neonatal research

The use of piglets as an animal model in human neonatal research has been steadily increasing over the last few decades. This increased interest in piglets as an animal model for human diseases has been based mainly on the similarities between the two species. The neonatal piglet is considered an appropriate model for the human infants because of the comparative anatomy, physiology and metabolism (Benevenga et al., 1986; Moughan et al., 1992). As a result, neonatal piglets are used as general surgical models of most organs and systems, for

Table 2.5 Previous animal studies on aluminum and/or TPN in PNAC

Studies	Animal	Factor	Dose	Duration	Result
Klein et al. 1987	piglets, 6 weeks old	aluminum	1.5 $mg \cdot kg^{-1} \cdot day^{-1}$	50 days	Cholestasis
Duerkse et al. 1996	newborn piglets	TPN	unknown	21days	Decrease bile flow and elevated serum total bilirubin concentration
Demircan et al. 1998	rats	aluminum and/or TPN	66.85 $\mu g \cdot kg^{-1} \cdot day^{-1}$	7 days 14 days	Portal inflammation
Klein et al. 1988	rats	aluminum	1 $mg \cdot kg^{-1} \cdot day^{-1}$ 5 $mg \cdot kg^{-1} \cdot day^{-1}$	7 days 14 days	Hepatobiliary dysfunction
Gonzalez et al. 2004	rats	aluminum	27 $mg \cdot kg^{-1}$, 3 times a week	90 days	Cholestasis

cardiovascular research, for digestive system models, and in recent years in transplantation and xenografic research. In the nutrition research area, the neonatal piglets has been widely used as a model for studying nutrient requirements (Reeds and Odle, 1996; Jahoor et al., 1999) and digestive function in human infants (Donovan et al., 1996), and are also used to monitor amino acid metabolism and gastrointestinal function during total parenteral nutrition (TPN) (Wykes et al., 1993; Ball et al., 1996) as well as the hepatotoxicity of TPN (Cohen et al., 1990; Duerksen et al., 1996). They also have been used as a replacement for rats, or other experimental animals in some research. Not only the regulatory pressure on other large animal species, but also because piglets are recognized as a suitable animal model for human diseases, the rise in using piglets in human neonatal research will probably continue.

2.5.1 The similarities of porcine and human infants

Many anatomical and physiological similarities between humans and pigs have been documented (Pond and Mersmann, 2001). The predominant porcine systems studied in biomedical research are cardiovascular, digestive, dermal and urinary.

Swine are true omnivores. The general architecture of the gastrointestinal tracts of the pig and human is similar. Notable differences are the higher proportion of cardiac mucosa lining the stomach of the pig, as compared with the human (Yen, 2001), and the presence of a cecum in the pig, as compared with only a vestige, the appendix, in the human. The muscular layers of the esophagus are mainly composed of smooth muscle until its termination cranial to the esophageal sphincter

when it becomes partially striated muscle. The stomach is typical of monogastric animals with the exception of the torus pyloricus, which is a muscular outpouching near the pyloric sphincter. The small intestine is long and located mainly in the right side of the abdomen. The majority of the large intestine is located in the spiral colon in the left upper quadrant of the abdomen. It consists of the cecum, ascending, transverse and a portion of the descending colon coiled tightly into a series of centripetal and centrifugal coils. The liver contains six lobes and a gall bladder. The common bile duct enters the duodenum separately from the pancreatic duct caudal to the pylorus. The pancreas is extensive and the tail follows the lesser curvature of the stomach from the spleen to the proximal duodenum. The pancreatic ducts in the tail and body join at the juncture of the two lobes to enter the duodenum distal from the bile duct. Functionally both the liver and pancreas are similar to human (Ford et al. 2001; Schantz et al., 1996).

Early postnatal growth of the pig is characterized by a period of rapid growth. During the first week after birth, a well-fed pig can double its birth weight. At birth the body of the pig contains only minimal amounts of fat (usually considered to be no more than 2% of body weight); however, the rapid growth is accompanied by a dramatic increase in fat deposition, and within 1 week the fat content can increase by 10 to 20-fold. Since the baby pig has limited capacity for fatty acid synthesis, most of this fat is deposited from the dietary source of preformed, long chain fatty acids. The relative rates of protein deposition and lean growth are very high (Wood and Groves, 1965). The growth and changes in body composition in pigs that occur between birth and weaning at age 4 weeks are summarized in Figure 2.4 (Manners and McCrea, 1963; Mitchell et al., 2001).

Compared with the human being, the piglets develop and grow more rapidly

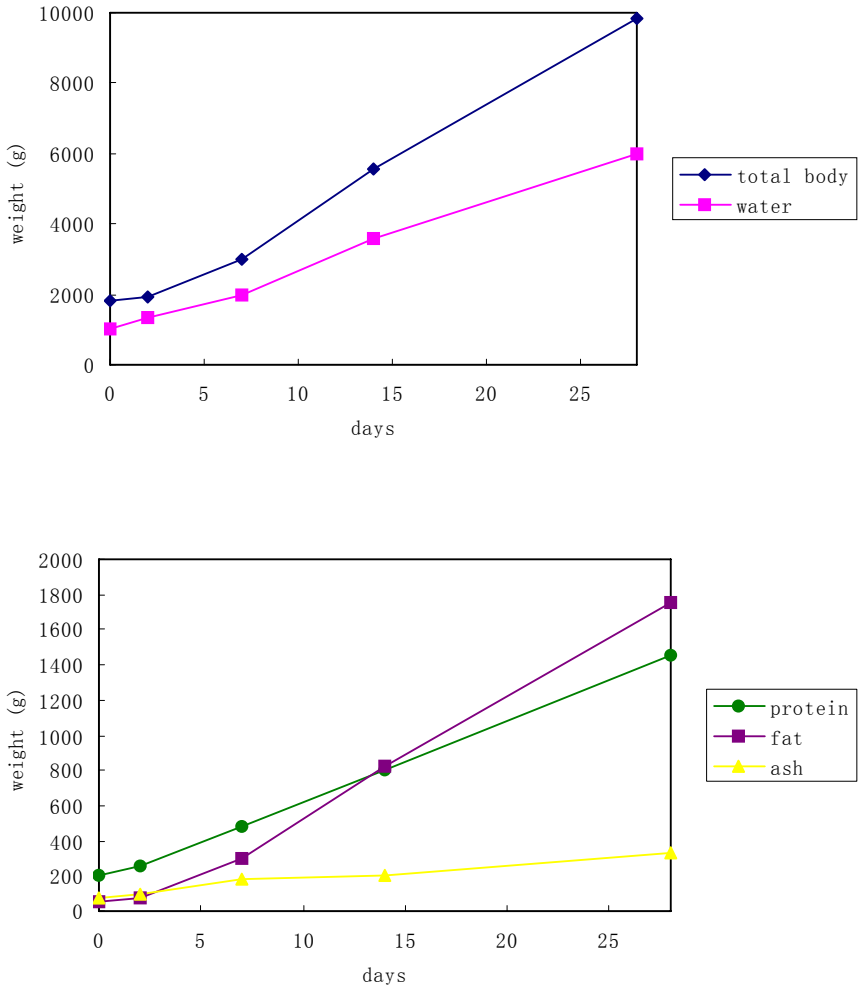


Figure 2.4 Growth and body composition of the pig from birth to age 4 weeks (Manners and McCrea, 1963; Mitchell et al., 2001)

than the human infant, but this can be viewed as an accelerated model of postnatal growth and development (Mount and Ingram, 1971). This rapid growth may heighten the piglet's sensitivity for the detection of metabolic responses to different changes in the body (Wykes et al., 1993). The newborn piglet contains <2% body fat, in contrast to the full-term human infant with an average fat content of 10%. The newborn piglets resemble the low-birth-weight human infant in fat content. Therefore, the piglet is increasingly used as a model for studies of alimentation of premature infants, especially in lipid metabolism.

2.5.2 The comparison of piglet model and human infants in parenteral nutrition associated cholestasis

Parenteral nutrition is very important for human infants who cannot tolerate enteral feeding. The piglet and humans have nearly identical gastrointestinal, hepatic, and hemopoietic physiology. Nutrient requirements, growth rates and dentition are also comparable (Burrin, 2001) (Table 2.6).

For these reasons, more and more researches choose the neonatal piglets as the animal model for human neonatal research in PN therapy. Cohen et al. (1986) placed 53 piglets with a mean weight of 1.8 kg and a mean age of 3.1 days on TPN for periods ranging from 6 to 31 days. Clinical complications were similar to those observed in human infants on PN therapy. It has been possible to maintain piglets with a central venous catheter for significant periods of time (up to 50 days) safely and effectively (Klein et al., 1987). In recent studies, piglets are used as the animal model in evaluating the nutrients in parenteral feeding formula, contaminators in PN solution and the complications of PN therapy in infants (Van-Aerde et al., 1999; Bertolo et al., 1999; Duerksen et al., 1996; Dahlke et al., 2004).

Table 2.6 Comparison of nutrient requirements of the human infant and the piglet

Nutrient	Piglet Requirement[1] (unit·kg^{-1}·d^{-1})	Human infant Requirement[2] (unit·kg^{-1}·d^{-1})	Ratio piglet: human infant
Energy (kcal)	205	108	1.9
Protein (g)	16.25	1.52	10.7
Sodium (mg)	158	ND[3]	ND[3]
Calcium (mg)	563	35	16.1
Phosphorus (mg)	438	16.7	26.2
Chlorine (mg)	158	20.8	7.6
Magnesium (μg)	250	5000	0.05
Potassium (mg)	188	ND[3]	ND[3]
Copper (mg)	0.375	0.033	11.4
Iodine (μg)	10	18.3	0.6
Iron (mg)	6.25	0.045	138.9
Manganese (μg)	250	0.5	500
Selenium (μg)	20	2.5	8
Zinc (mg)	6.25	0.33	18.9
Vitamin A (μg)	41.3	66.7	0.6
Vitamin D (μg)	0.34	0.83	0.4
Vitamin E (μg)	0.67	0.67	1
Vitamin K (μg)	32.5	0.33	98.5
Vitamin C (mg)	ND[3]	6.67	ND[3]
Biotin (μg)	5	0.83	6
Choline (mg)	37.5	20.8	1.8
Folate (μg)	20	10.8	1.9
Niacin (mg)	1.25	0.33	3.8
Pantothenic acid (μg)	750	283	2.7
Riboflavin (μg)	250	50	5
Thiamin (μg)	95	33.3	2.9
Vitamin B_6 (μg)	125	16.7	7.5
Vitamin B_{12} (μg)	1.25	0.067	18.7
Linoleic acid (mg)	62.5	0.73	85.6

1. Calculated from nutrients requirement for 3-5 kg piglets (NRC, 1998).
2. Calculated from nutrients requirement for 0-6 months infant (National Academy of Sciences, 2002).
3. Not determined.

In liver diseases research, because of the similarities of the anatomy and physiology of liver and bile secretion, piglet also is used as an animal model to represent the hepatobiliary function in the human infants. A comparative study of the hepatic bile ducts of human and laboratory animals was made by means of scanning electron microscopy of biliary tract casts (Yamamoto et al., 1985). In the pig, many pouches are observed around not only large but also small bile ducts, which is similar to the human being. No such structures are observed in dog, guinea pig, or rabbit bile ducts. Many suitable piglet models are developed to demonstrate the different characteristics of liver function in human infants (Desport et al., 1997; Truskett et al., 1987; Dahlke et al., 2004).

For the reasons stated, the piglet model is suitable and useful for studying PNAC in infants. Development of suitable models in piglets could be valuable in the understanding and prevention of PNAC in human infants.

3. EXPERIMENTS

3.1 The role of aluminum in parenteral nutrition associated cholestasis in human infants: a prospective study

3.1.1 Introduction

Aluminum is a known contaminant of PN solutions (Klein, 2003). Elevated aluminum level in urine, blood, liver, bone and brain has been documented in patients on PN (Sedman et al., 1985; Koo et al., 1986; Moreno et al., 1994). Despite attempts by several investigators, the role of aluminum in PNAC remains obscure. The safe aluminum levels for the infants on PN are not established (Klein, 2003). Some questions concerning aluminum exposure, the prematurity of the infants, and the whole process of developing PNAC need to be defined.

The study was designed to investigate the relationship between the serum aluminum levels and the direct bilirubin concentrations in gastrointestinal failure infants who required PN. The hypothesis of the study is the increased serum aluminum concentration would be correlated with increased serum direct bilirubin in PNAC infants. Other potential factors may also play roles in this relationship.

3.1.2 Methods

3.1.2.1 Subjects

Patients were recruited from the neonatal intensive care unit (NICU) at the Royal University Hospital (RUH), Saskatoon, Saskatchewan, Canada. Eligible

patients were infants, age <365 days who meet inclusion criteria of anticipating to require PN therapy for 3 weeks secondary to the diagnosis of gastrointestinal failure. Patients were excluded if they were diagnosed with the following diseases:

1. congenital hepatic or biliary disorder such as: extrinsic obstruction of the biliary system, hepatic neoplasm, inspissated bile syndrome, spontaneous perforation of the common bile duct, infantile obstructive cholangiopathy (neonatal hepatitis, biliary atresia, biliary hypoplasia, choledochal cyst, Alagille's syndrome)
2. major cardiac anomaly
3. other congenital or acquired disorders which may cause hepatic disease such as: hepatitis, syphilis, rubella, toxoplasmosis, metabolic disorders (i.e., galactosaemia, fructosaemia, alpha-1-antitrypsin deficiency, cystic fibrosis) and endocrine disorders (i.e., hypothyroidism, hypopituitarism, hypoadrenalism)

All subjects enrolled received the pediatric PN solutions (see the components of PN solutions in Table 3.2 in the results) made by the Department of Pharmaceutical Service in the RUH. From a review of health records, we have identified infants with intestinal failure from necrotizing enterocolitis, gastroschisis, omphalocele, intestinal atresia, and intestinal aganglionosis as likely to require PN for at least 3 weeks.

3.1.2.2 Design

Patients were enrolled by a research team in NICU at the RUH. Eligible patients were followed during the PN therapy. The anticipated period for the study was 3 weeks. During the study period, blood samples were taken from the patients

once a week and analyzed for the aluminum levels, liver enzymes, and bilirubin. The diagnosis of PNAC was made when the serum direct bilirubin was greater than 34 $\mu mol \cdot L^{-1}$ during the PN therapy (Moreno et al., 1994; Hata et al., 1993; Beath et al., 1996; Kubota et al., 2000; Teitelbaum et al., 2001). The research protocol, including the consent process and form has been approved by the University of Saskatchewan Advisory Committee on Ethics in Human Experimentation and the Saskatoon District Health Ethics Committee (Appendix A). Informed consent for participation in the study will be obtained from the subject's legal guardian. See appendices for the study protocol (Appendix B) and consent form (Appendix A).

3.1.3 Sample collection and analysis

Eligible patients' basic information and PN solution intake were obtained from their hospital records. Daily weights were recorded by the nurse in the NICU. At day 0, day 7, day 14 and day 21, 5 mL blood was collected in the metal free tubes from each enrolled patient by phlebotomy personnel at the Royal University Hospital. The serum was analyzed for serum biochemistry including alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and bilirubin (direct and indirect bilirubin) as well as aluminum evaluation. Serum biochemistry was determined on a Beckman-Coulter LX-20 by standard enzymatic methods (Fody E.P., 2000) at the Department of Laboratory Medicine in RUH. Aluminum level was determined at the Saskatchewan Research Council (SRC), Saskatoon, Canada, by inductively coupled plasma atomic emission spectrometry (ICP-AES). See Appendix D for the quality control of aluminum analysis.

3.1.4 Statistical methods

Descriptive statistics was performed on the raw data. Results are expressed as mean ± standard deviation (SD). Two-tailed Pearson correlation is performed to test the relationship of serum aluminum and serum bilirubin. PNAC patients are compared with non-PNAC patients by unpaired t-tests. Values are considered significant at $p<0.05$.

3.1.5 Results

3.1.5.1 General

A total of sixteen patients were enrolled (Table 3.1). There were ten males and six females in the study. These patients were diagnosed with intestinal atresia (n=8), gastroschisis (n=7) and meconium peritonitis (n=1). The birth weight was 2426±795 g (900g to 3627 g). The average gestation age was 35.4±4.0 weeks (26 to 42 weeks). The duration of PN therapy was 16.2±7.9 days (7 to 35 days).

Aluminum content in each component of the PN solution was measured. The aluminum concentration, based on the standard hospital formulation for infants was calculated at 252.6 $\mu g \cdot L^{-1}$ (Table 3.2). The volume of fluids intake was obtained from the hospital records, and the mean daily aluminum received from PN in these infants was estimated at 21.6±5.2 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (Table 3.3).

3.1.5.2 PNAC group versus non-PNAC group

Five of sixteen (31.3%) patients developed PNAC, which was diagnosed when serum direct bilirubin was greater than 34 $\mu mol \cdot L^{-1}$. There were no significant differences between the PNAC and non-PNAC group in birth weight,

Table 3.1 The gender, birth weight, gestation age, duration of PN and diagnosis of the enrolled infants

Subject number	Gender	Birth weight (g)	Gestation age (weeks)	Duration of PN (days)	Diagnosis
PNAC subject number					
1	male	1510	31	19	Intestinal atresia
3	male	1670	32	12	Intestinal atresia
4	male	2380	35	12	Gastroschisis
11	male	2290	36	21	Intestinal atresia
15	male	3120	38	16	Meconium peritonitis
PNAC Subtotal (n=5)	**male (n=5) female (n=0)**	**2194±641**	**34.4±2.9**	**16.3±4.1**	**Intestinal atresia (n=3) Gastroschisis (n=1) Meconium peritonitis (n=1)**
Non-PNAC subject number					
2	male	3627	40	7	Intestinal atresia
5	female	3050	36	10	Gastroschisis
6	female	3030	38	15	Gastroschisis
7	female	2720	37	14	Gastroschisis
8	female	2540	38	9	Intestinal atresia
9	female	1700	33	10	Intestinal atresia
10	female	2470	35	25	Gastroschisis
12	male	900	26	35	Intestinal atresia
13	male	3300	42	13	Intestinal atresia
14	male	3150	39	11	Gastroschisis
16	male	1360	31	30	Gastroschisis
Non-PNAC Subtotal (n=11)	**male (n=5) female (n=6)**	**2531±863**	**35.9±4.5**	**16.0±9.4**	**Intestinal atresia (n=5) Gastroschisis (n=6)**
Total (n=16)	**male (n=10) female (n=6)**	**2426±795**	**35.4±4.0**	**16.2±7.9**	**Intestinal atresia (n=8) Gastroschisis (n=7) Meconium peritonitis (n=1)**

1. Data of birth weight, gestation age and duration of PN in subtotal and total were expressed as mean ± SD.

Table 3.2 Aluminum concentrations in parenteral solutions used in the hospital

Product	Aluminum concentration ($\mu g \cdot L^{-1}$)	Pediatric PN solution[11] ($mL \cdot L^{-1}$)	Aluminum[13] (μg)
Pediatric amino acid 10%[1] (Aminosyn PF)	<50[12]	250	6.3
Sterile water[2]	2.2	557	1.2
Dextrose 70%[3]	<50[12]	125	3.1
Potassium phosphate[4]	7900	3	23.7
Potassium acetate[5]	<50[12]	0.3	0.0
Calcium gluconate[6]	4200	51.6	216.7
Zinc sulfate[7]	400	2.13	0.9
Sodium chloride 23.4%[8]	<100[12]	2.8	0.1
Sodium acetate[9]	60	7.5	0.5
Magnesium sulfate 50%[10]	<300[12]	0.72	0.1
Total		1000	252.6

1. One mL contains 100 mg amino acids. Baxter Corporation, Mississauga, Ontario, Canada.
2. Baxter Corporation, Mississauga, Ontario, Canada.
3. One mL contains 700 mg dextrose. Baxter Corporation, Mississauga, Ontario, Canada.
4. One mL contains 224 mg potassium phosphate monobasic, and 236mg potassium phosphate dibasic. Pharmaceutical Partners of Canada Inc., Richmond Hill, Ontario, Canada.
5. One mL contains 196.28 mg potassium acetate. Pharmaceutical Partners of Canada Inc., Richmond Hill, Ontario, Canada.
6. One mL contains 94 mg calcium gluconate, 4.5mg calcium saccarate. Pharmaceutical Partners of Canada Inc., Richmond Hill, Ontario, Canada.
7. One mL contains 21.95 mg zinc sulfate. Sabex 2002 Inc., Boucherville, Quebec, Canada.
8. One mL contains 234 mg sodium chloride. Sabex 2002 Inc., Boucherville, Quebec, Canada.
9. One mL contains 164 mg sodium acetate. Pharmaceutical Partners of Canada Inc., Richmond Hill, Ontario, Canada.
10. One mL contains 500 mg magnesium sulfate. Pharmaceutical Partners of Canada Inc., Richmond Hill, Ontario, Canada.
11. The formula was obtained from the Department of Pharmaceutical Service in RUH.
12. When the concentration was under the detected limitation, half of the value was used in the calculation.
13. The calculation of aluminum is: Aluminum concentration ($\mu g \cdot L^{-1}$) X Pediatric PN solution ($mL \cdot L^{-1}$) / 1000 = Aluminum (μg).

Table 3.3 An example of the calculation of parenteral aluminum intake from the PN therapy[1]

PN therapy	Aluminum concentration ($\mu g \cdot L^{-1}$)	Intake during PN therapy ($mL \cdot kg^{-1} \cdot day^{-1}$)	Parenteral aluminum intake[7] ($\mu g \cdot kg^{-1} \cdot day^{-1}$)
Pediatric PN solutions	252.6[5]	85.9	21.7
Intralipid 20%[2]	100[6]	11.9	1.2
Pediatric multivitamins[3]	220[6]	2	0.4
Pediatric trace elements[4]	350[6]	0.05	0
Total			23.3

1. The mean of intake ($mL \cdot kg^{-1} \cdot day^{-1}$) was calculated from the hospital records. Data in this table were from one of the enrolled infants (subject number 8)
2. One mL contains 20 mg lipids. Fresenius Kabi AB, Uppsala, Sweden.
3. One mL contains 460 IU vitamin A, 16 mg vitamin C, 80 IU vitamin D, 0.24 mg thiamine, 0.28 mg riboflavin, 0.2 mg pyridoxine, 3.4 mg niacinamide, 1.0 mg pantothenic acid, 1.4 IU vitamin A, 4 μg biotin, 28 μg folate, 0.2 μg vitamin B_{12}, and 40 μg vitamin K. Sabex 2002 Inc., Boucherville, Quebec, Canada.
4. One mL contains 3.0 mg zinc, 0.1 mg manganese, 0.4 mg copper, 4.0 μg chromium, 20 μg selenium, and 60 μg iodine. Sabex 2002 Inc., Boucherville, Quebec, Canada.
5. The value is from the total aluminum in the PN solution in Table 3.2
6. The concentrations of aluminum were determined at Saskatchewan Research Council (SRC), Saskatoon.
7. The calculation of parenteral aluminum intake is: Aluminum concentration ($\mu g \cdot L^{-1}$) X Intake during PN therapy ($mL \cdot kg^{-1} \cdot day^{-1}$) = Parenteral aluminum intake ($\mu g \cdot kg^{-1} \cdot day^{-1}$).

gestation age, and duration of PN therapy.

The mean serum ALP and GGT for both groups remained in the normal range throughout the study (Figure 3.1, Figure 3.2). The mean serum total bilirubin peaked at day 7 in both PNAC infants and non-PNAC infants (Figure 3.3). There were no significant differences between these two groups in the mean serum total bilirubin on day 0, day 7, day 14 and day 21.

Thirteen of sixteen infants in the study developed direct hyperbilirubinemia. Five infants developed PNAC based on a serum direct bilirubin $\geq 34\ \mu mol \cdot L^{-1}$. Both the PNAC and non-PNAC groups had similar serum direct bilirubin concentrations at the baseline. However, the PNAC group demonstrated a linear increase ($r^2=0.481$, $p<0.01$) while the non-PNAC group's values did not increase during the experiment (Figure 3.4).

The mean parenteral aluminum intake of both groups demonstrated a similar trend, starting with the greatest value at the baseline with steady decline over time (Figure 3.5). The similar trend of the mean serum aluminum concentration was shown in both the PNAC and non-PNAC infants (Figure 3.6). The concentrations peaked at day 7 and then declined thereafter. There were no significant differences between two groups in serum aluminum concentrations at day 0, day 7, day 14 and day 21. There was no significant correlation between the serum direct bilirubin concentrations and the serum aluminum levels in these infants ($p>0.05$).

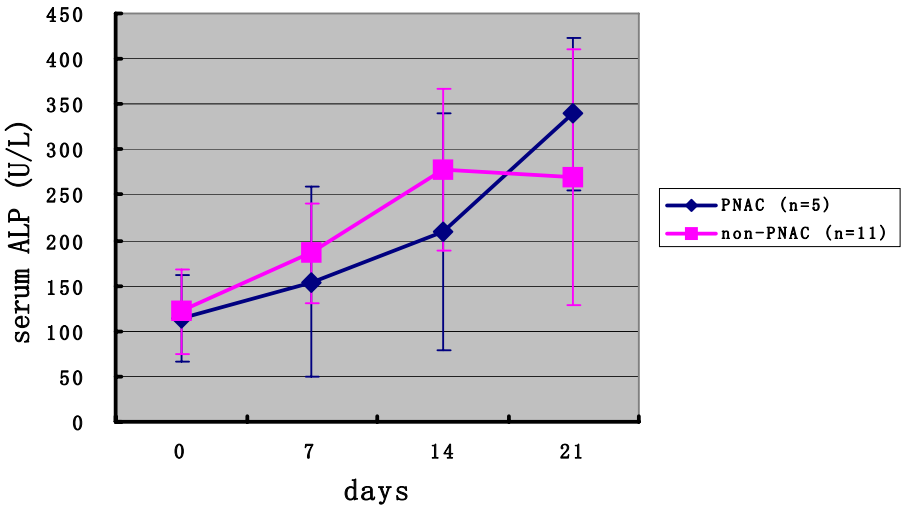


Figure 3.1 Serum ALP in the PNAC infants versus non-PNAC infants

The symbols (■ and ♦) represent means. Error bars are SD. n=8 for non-PNAC group at day 14. n=3 for PNAC group and n=6 for non-PNAC group at day 21. The reference value of serum ALP is from 150 to 500 $U \cdot L^{-1}$.

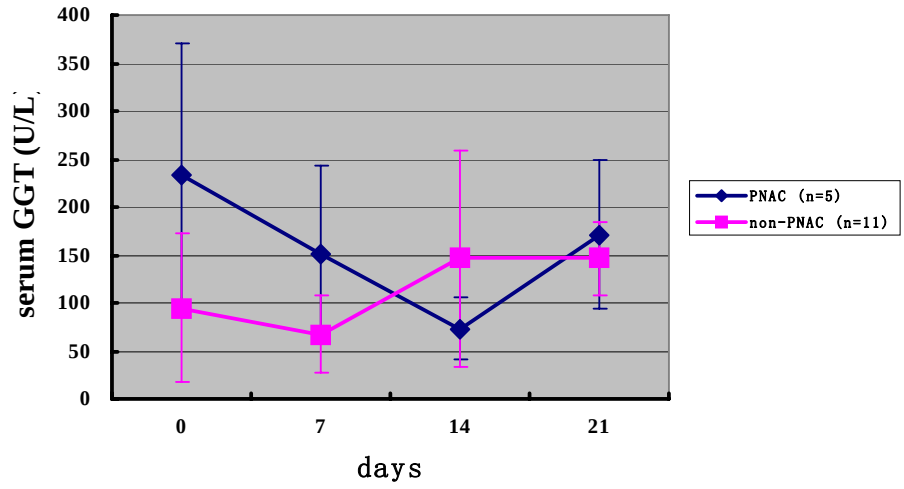


Figure 3.2 Serum GGT in PNAC infants versus non-PNAC infants

The symbols (■ and ♦) represent means. Error bars are SD. n=8 for non-PNAC group at day 14. n=3 for PNAC group and n=6 for non-PNAC group at day 21. The reference value of serum GGT is from 20 to 200 $U \cdot L^{-1}$.

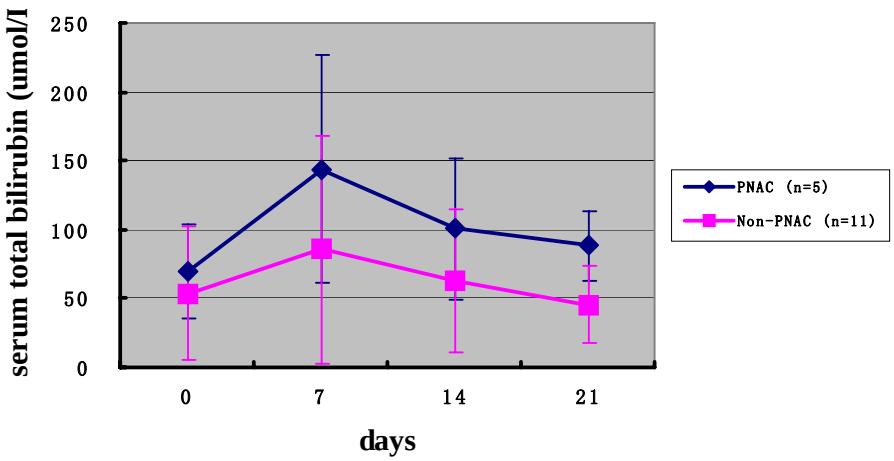


Figure 3.3 Serum total bilirubin in PNAC infants versus non-PNAC infants

The symbols (■ and ♦) represent means. Error bars are SD. n=8 for non-PNAC group at day 14. n=3 for PNAC group and n=6 for non-PNAC group at day 21. The reference value for serum total bilirubin is from 2 to 22 μmol·L^{-1}.

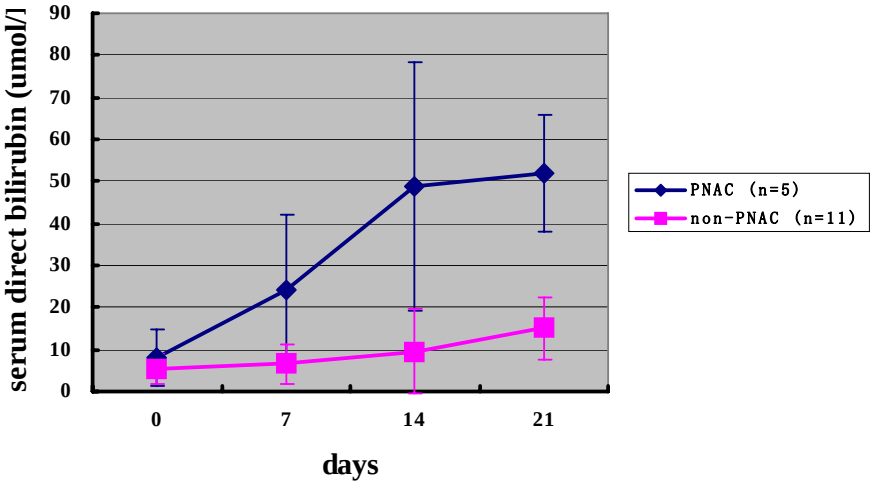


Figure 3.4 Serum direct bilirubin in the PNAC infants versus the non-PNAC infants

The symbols (■ and ♦) represent means. Error bars are SD. n=8 for non-PNAC group at day 14. n=3 for PNAC group and n=6 for non-PNAC group at day 21. A linear trend is shown in PNAC group (r^2=0.481, p<0.01). The reference value for serum dirct bilirubin is from 0 to 5 μmol·L^{-1}.

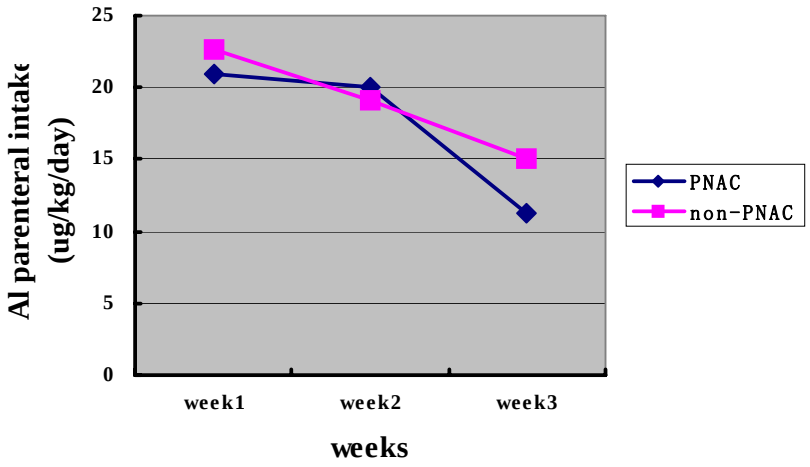


Figure 3.5 The mean parenteral aluminum intake in the PNAC group versus the non-PNAC group

The symbols (■ and ♦) represent means. The calculation of parenteral aluminum intake was demonstrated in Table 3.3. n=5 (PNAC group) and n=11 (non-PNAC group) for week1. n=5 (PNAC group) and n=8 (non-PNAC group) for week2. n=3 (PNAC group) and n=6 (non-PNAC group) for week3.

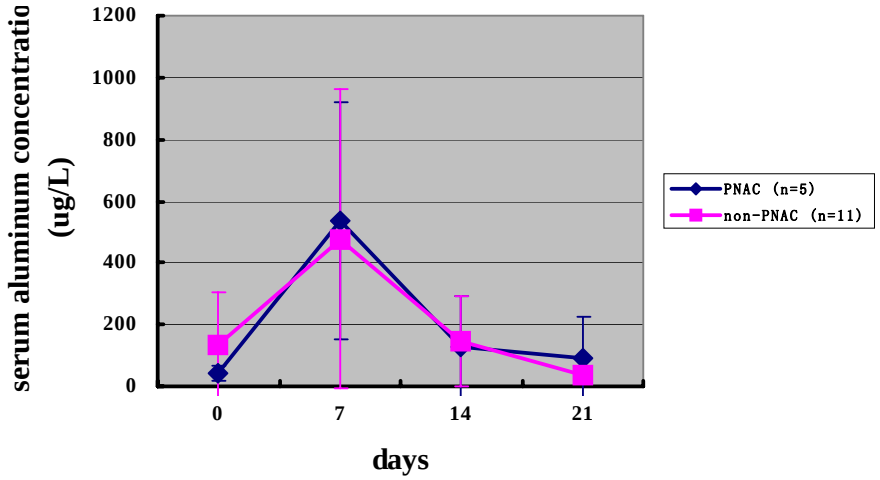


Figure 3.6 Serum aluminum concentrations in the PNAC infants versus the non-PNAC infants

The symbols (■ and ♦) represent means. Error bars are SD. When the concentration was under the detected limitation, half of the value was used in the calculation. n=8 for non-PNAC group at day 14. n=3 for PNAC group and n=6 for non-PNAC group at day 21.

3.1.6 Discussion

The incidence of PNAC was 31.25% (5 of 16). In our unpublished retrospective data (Arnold, 2004) the incident rate of PNAC was 25%, which was in agreement with Kubota's work (Kubota et al., 2000). The variation in reported incidence is due to differences in study populations, diagnosis of cholestasis, duration of the study time, and other medical or surgical conditions in the study subjects (Btaiche and Khalidi, 2002).

There was no statistical difference in birth weight between the PNAC and non-PNAC infants. Previous human studies suggested that PNAC was more common in extremely low birth weight infants (Beale, et al., 1979; Drongowski and Coran, 1989; Kelly, 1998). In Beale's work, the infants with birth weight under 1000 g had an incidence of 50% (Beale et al., 1979). In our unpublished retrospective study, results showed that birth weight less than 1600 g significantly contributed to the development of PNAC (Arnold, 2004). It is important to point out only one infant had a birth weight under 1000 g, and three had a birth weight less than 1600 g in this prospective study. The reason this study did not show a significant difference may be due to the small size of the sample. Other human studies also showed mixed results regarding the role of birth weight in PNAC. There is no agreement on the cut-off line for low birth weight infants who may have a high risk for PNAC (Beale, et al., 1979; Drongowski and Coran, 1989; Kelly, 1998). Gestation age is also related to birth weight. Note that only one infant in the study had a gestation age lower than 30 weeks. Our unpublished retrospective study

suggested that PNAC infants were born at relatively the same gestation age yet were significantly lighter (Arnold, 2004). This may explain the no difference showed in gestational age between the PNAC group and the non-PNAC group. Both birth weight and gestation ages are markers of maturity of infants at birth. Most of the investigators agreed that the premature infants were on the high risk of PNAC (Btaiche and Khalidi, 2002; Beath et al., 1996). However, when birth weight or gestation age were used as the markers of prematurity, mixed results were found in the previous human studies (Baserga and Sola, 2004; Zambrano et al., 2004; Drongowski and Coran, 1989).

The duration of PN is another potential factor of PNAC development in infants (Gremse and Balistreri, 1989). No significant difference was found in duration of PN in the study. This agreed with Beath's work, which suggested that the duration of PN did not correlate significantly with the development of cholestasis (Beath et al., 1996). However, it is important to note that the small number of the patients in our study may affect the difference between PNAC and non-PNAC infants. Moreover, the experimental period was only 3 weeks. It may be different in the patients who required long term PN therapy.

Serum ALP and GGT are used as indicators of cholestastic disease. Both serum ALP and GGT were within the normal range. However, serum direct bilirubin had already increased, which indicated the development of PNAC. Therefore, serum ALP and GGT may not be a sensitive indicator for PNAC.

The mean total bilirubin and direct bilirubin concentrations in the study are

clinically different. The mean total bilirubin of all 16 infants demonstrated a steady increase from baseline peaking on day 7 and then a steady decline approaching normal. The mean indirect bilirubin had the similar trend as the mean total bilirubin. This trend was considered as physiological jaundice of the newborn (Hansen, 2000). For the mean direct bilirubin, the PNAC group demonstrated a linear increase while non-PNAC group's values did not increase while on PN. Since there were no significant difference in birth weight, gestation age and the duration of PN between these two groups, other factors would be responsible for the significant difference in the mean direct bilirubin. Serum direct bilirubin was the indicator and diagnosis standard for PNAC. PNAC was diagnosed when the serum direct bilirubin was greater than 34 $\mu mol \cdot L^{-1}$, which was much higher than the normal value (0 to 5 $\mu mol \cdot L^{-1}$). The diagnosis standard was adapted from the previous human studies (Moreno et al., 1994; Hata et al., 1993; Beath et al., 1996). However, the diagnostic standard for PNAC used in the study was relatively arbitrary. It was interesting to find that there was 9 infants developed PNAC if the diagnosis standard for PNAC was the serum direct bilirubin greater than 12 $\mu mol \cdot L^{-1}$. Moreover, these PNAC infants (n=9) would have a significant lower birth weight ($p<0.01$) and gestation age ($p<0.01$) compared with the non-PNAC infants (n=7). Note that thirteen of sixteen infants developed conjugated hyperbilirubinemia during the PN therapy. Conjugated hyperbilirubinemia occurs when bilirubin is returned to the bloodstream after conjugation in the liver, instead of draining into the bile ducts. The most common causes are hepatocellular disease, intrahepatic cholestasis and extrahepatic

obstruction (Hansen, 2000). The hepatobiliary dysfunction is one of the most common complications of PN therapy.

For infants who receive PN, the major source, and in many cases the only source of aluminum intake is the contaminant aluminum in PN solutions (Moreno et al., 1994). Different products, even different lot numbers may also contain the different contamination levels of aluminum in PN solutions (Klein, 2003). The amount of aluminum in PN solutions may vary from different reports. In Moreno's study, the Al contamination level was about 10.6 to16.7 $\mu g \cdot kg^{-1} \cdot day^{-1}$ for the infants on PN (Moreno et al., 1994). Another study in Alberta, Canada showed that the level ranged from 17.9 to 38.3 $\mu g \cdot kg^{-1} \cdot day^{-1}$ for the neonatal patients on PN (Koo et al., 1986). The mean parenteral intake of aluminum in the total 16 patients was 21.6 $\mu g \cdot kg^{-1} \cdot day^{-1}$. Similar parenteral aluminum intake was found in both PNAC and non-PNAC infants. It is important to note that this aluminum level in PN solutions significantly exceeds the 2 $\mu g \cdot kg^{-1} \cdot day^{-1}$ safe level that is recommended for adult (ASCN/ASPEN, 1991). The declined level of parenteral aluminum intake in the third week (Figure 3.5) was because most of the subjects decreased the intake from the PN solutions and started the oral intake in the third week of the study.

Serum aluminum peaked on day 7 and then declined after in both the PNAC and non-PNAC group. This fall after 7days may be due to further maturity of the renal system in these patients, which thus increases the elimination of aluminum. The parenteral aluminum intake was not correlated with serum aluminum levels. The variability of excretion and deposition would explain why these infants had the

same aluminum intake but different serum aluminum levels. Although full-term infants have better renal function than premature newborns, they also have a reduced glomerular filtration rate compared with adults and do not reach normal values until 1-2 years of age (Engle, 1986). Thus, with the unsafe level of parenteral aluminum intake, combined with low aluminum excretion ability or high aluminum deposition in the liver may put the infants who required PN at a high risk of PNAC. In the United Sates, legislation was made by the Food and Drug Administration to limit the amount of aluminum allowable in intravenous solutions (FDA, 2000).

There was no positive relationship between serum aluminum and serum direct bilirubin. Serum aluminum is not a good marker of PNAC. The peak of serum aluminum was at day 7 although the serum direct bilirubin kept climbing up to day 21 in the PNAC infants. The result may be due to the accumulation of aluminum in the liver and further damage the ability to excrete aluminum. In the previous human studies, elevated serum aluminum levels were found in the PNAC infants (Moreno et al., 1994; Koo et al., 1986). However, there has never been any proven relationship between serum aluminum and degree of cholestasis in humans (Klein, 2003).

One limitation of this research is that the medications that could cause cholestasis were not accounted for, such as anabolic steroids, chlorpromazine, prochlorperazine, sulindac, cimetidine, ampicillin, and other penicillin-based antibiotics. However, most of these drugs are rarely used in the neonatal patients. For parenteral aluminum intake, there may be variation from batch to batch in PN

solutions. It was not practical and worthwhile to check the aluminum level of every bag. The estimated aluminum level in the study is close to other reports which may use similar products (Koo et al., 1986; Moreno et al., 1994; Klein, 2003). Although the intravenous drugs may also contribute to the aluminum level, they only represent 1.7% of total parenteral aluminum intake (Moreno et al., 1994). Therefore, the aluminum in PN solution may be responsible for the aluminum loading of the infants. The major aluminum contributor in PN solution was electrolytes (see Table 3.2). The different concentrations of electrolytes other than the formulation may be used in the PN solution for the individual patients. Thus, the different aluminum concentrations in the PN solutions for different patients weren't accounted for. Serum aluminum level could only reflect aluminum exposure. The elimination of aluminum was not assessed. Moreover the small sample size of this study may hinder statistical comparison between the PNAC and non-PNAC group.

3.1.7 Conclusion

PNAC has been shown to develop in less than three weeks in the infants with gastrointestinal failure who required PN therapy. Despite the similar parenteral aluminum intake, PNAC (serum direct bilirubin level $\geq 34\ \mu mol \cdot L^{-1}$) only occured in 5 patients. Serum aluminum level had a peak on day7 then declined thereafter, and it was not directly correlated with parenteral aluminum intake or serum direct bilirubin.

It has been suggested that the cause of PNAC in infants are multifactorial

(Balistreri et al., 1983; Merrit, 1986; Klein, 2003). The variability of each potential factor that contributes to the disease in individual infants is high. The significant exposure to aluminum in the infants who required PN therapy can cause its accumulation in organs leading to toxicity. The aluminum contaminant in PN solutions has been implicated as one of the potential factors in the development of PNAC. However, the mechanism of aluminum in the etiology of PNAC is still unclear. And the contribution of aluminum in this multifactor model and the relationship with other potential factors requires further exploration.

3.2 The role of aluminum in parenteral nutrition associated cholestasis in neonatal piglets: an experimental model study

3.2.1 Introduction

Human infant study is constrained by several factors, including: a heterogeneous population, ethical and practical limitations, and acceptable methods design. Therefore, the application of an animal model permits experimental study which can provide a comprehensive and in-depth picture of the role of aluminum in the development of PNAC. Several studies have shown that aluminum administered to rats and piglets in experimental pharmacologic doses produces cholestasis (Klein et al., 1988; Klein et al., 1987; Dermircan et al., 1998; Gonzalez et al., 2004). However, previous animal experiments used non-neonatal animals with aluminum doses far exceeding the level of contamination seen in the human patient care today.

A neonatal piglet model that used the same level of aluminum contamination as found in the hospital and imitated the infants on PN therapy was developed in this study. Techniques for central venous catheterization, sampling blood, patency of catheter, PN regimen delivery, and aluminum infusion were developed to facilitate the experiment. The overall objectives of the study were to compare different doses of intravenous aluminum in the development of cholestasis and to determine if PN therapy with contaminant aluminum is associated with the development of PNAC. Experimental procedures were approved by the University of Saskatchewan Committee on Animal Care and Supply Protocol Review Committee (Appendix A).

3.2.2 Materials and methods

3.2.2.1 Animals

Neonatal piglets were selected because of the comparative anatomy, physiology and metabolism between human infants and neonatal piglets, especially the similarities of liver function and bile secretion (Pond and Mersmann, 2000). Furthermore, the similar clinical complications to those observed in human infants on PN were found in the piglets (Tumbleson, 1986). Previous animal studies already demonstrated the safety and efficiency in keeping the catheter in the piglets up to 50 days and maintaining piglets on PN for 3 weeks (Klein et al., 1986; Duerksen et al., 1996).

3.2.2.2 Design

24 piglets, 2 to 4 days old, were obtained from the University of Saskatchewan Prairie Swine Center and placed into four groups: (1) Control group (n=5): daily intravenous injection saline (0.9% sodium chloride); (2) Low Al (aluminum) group (n=7): daily intravenous injection with aluminum dose at 20 $\mu g \cdot kg^{-1} \cdot day^{-1}$; (3) High Al (aluminum) group (n=6): daily intravenous injection with aluminum dose at 1500 $\mu g \cdot kg^{-1} \cdot day^{-1}$; (4) PN group (n=6): continuous PN solutions with aluminum intake at 37.8±14.3 $\mu g \cdot kg^{-1} \cdot day^{-1}$.

The aluminum content of saline (Abbott Laboratories, Montreal, Quebec, Canada) was lower than 1 $\mu g \cdot L^{-1}$ and has been used as control material in the previous aluminum studies (Klein et al., 1986; Klein et al., 1988; Demircan et al., 1998; Gonzalez et al., 2004). The aluminum injections in the Low Al and High Al groups were in the form of aluminum chloride hexahydrate (Fisher Scientific,

Mississauga, Ontario, Canada). See Appendix B for the preparation of aluminum injection. The aluminum level 20 $\mu g \cdot kg^{-1} \cdot day^{-1}$ in the Low dose group represents the contaminating level in the clinical PN therapy in infants (Moreno et al., 1994). The high dose aluminum 1500 $\mu g \cdot kg^{-1} \cdot day^{-1}$ was the dose used in the previous study for adult pigs for 50 days (Klein et al., 1986). The PN group received a balanced PN solution which was designed to, as closely as possible, achieve the estimated intravenous nutrient requirements of the piglet. All the solutions used in the PN regimen were the same commercial products used in the local hospital for infants. The amount of aluminum in these PN solutions was measured. The experiment time was 21 days.

3.2.2.3 Surgery

All operations were carried out with sterile technique in a Canadian Council on Animal Care certified operative facility at the Medical Research Building, University of Saskatchewan. The Subjects were given intramuscular (im) atropine (Glaxo Laboratories Inc., Toronto, Ontario, Canada) 0.09 $mg \cdot kg^{-1}$ prior to induction of anesthesia. Anaesthesia was induced with Ketamine (Rogarsetic, Rogar STB Inc., Montreal, Quebec, Canada) 22 $mg \cdot kg^{-1}$ im and Acepromaxine (Atravet, Ayerst Laboratories, Montreal, Quebec, Canada) 0.5 $mg \cdot kg^{-1}$ im. They were intubated and mechanically ventilated, with anesthesia maintained by isoflurane, administered nitrous oxide and oxygen (1:2). Excenel (3 $mg \cdot kg^{-1} \cdot day^{-1}$, im) (Pharmacia Animal Health, Missasaga, Ontario, Canada) was administered as a preoperative prophylactic antibiotic.

An incision was made on the right side of the neck and a 3 French (outside diameter) silicone implanted venous access catheter (Fisher Scientific, Mississauga,

Ontario, Canada) was inserted into the central venous system via an internal jugular vein and tunneled under the skin and exited on the animal's back. The animals were fitted with bandages to protect the catheter. For the piglets in the PN group, the end of the catheter was attached to a metal anchor and fixed into a special jacket (Lomir Biomedical Inc., Perrot, Quebec, Canada).

3.2.2.4 The care of piglets

The piglets were housed in a room with a 12-hour light/dark cycle. The room temperature was maintained at 27^{o} Celsius. The piglets were kept in individual cages with a secured feeder and water supply. Each cage had an adjustably positioned heat lamp. Excluding the PN group, the piglets had free access to food and water. PN solutions were administered through a tether-swivel system (Lomir Biomedical Inc., Perrot, Quebec, Canada). The swivel was secured at the top of the cage. The tether was attached to the anchor on the piglet's jacket. The system allowed the piglet freedom of movement without twisting or tangling the tubing. Each piglet had access to a toy for entertainment, and a blanket for resting.

All piglets were monitored daily for intake, weight gain, color of mucus membranes, feces and urine production. Cages were cleaned and the blanket changed daily. The patency of catheter was maintained by daily flushing with 0.9%sodium chloride solution (Abbott Laboratories, Montreal, Quebec, Canada). The incisions were cleaned daily until healed and the sutures were removed on postoperative day 7.

3.2.2.5 Parenteral nutrition (PN) formulation and PN regimen delivery

The PN formulation was designed to provide all nutrients required by the neonatal piglets. The intravenous nutrient requirements were estimated from the requirements of orally fed piglets (NRC, 1998) and the consideration of the role of the gastrointestinal tract in the metabolism of each nutrient. The estimated intravenous requirements are calculated in Table 3.4. Estimated requirement achieved by PN was shown in Table 3.5.

PN solutions were made everyday in a sterile lab in the College of Pharmacy and Nutrition, University of Saskatchewan. The procedure of PN solution compounding was discussed and approved by the clinical pharmacist. See the procotol of PN solution preparation in Appendix B. The commercial products used were the same products used in the local hospital (Table 3.6).

PN solutions were delivered by an infusion pump (Lifecare 5000, Abbott Laboratories, Chicago, USA) with pressure sensitive cassette tubing (Primary Pump Set, Abbott Laboratories, Montreal, Quebec, Canada). The lipid component of the PN was transferred to 30 or 60 mL syringes, and infused by syringe infusion pumps (Sage Pump, Model 365, Orion, USA). Rates were adjusted for daily weight gain to deliver 280 $mL \cdot kg^{-1} \cdot day^{-1}$ of PN solutions and 20 $mL \cdot kg^{-1} \cdot day^{-1}$ of lipids. PN was started approximately 3 hours after surgery. The rate started at 10 $mL \cdot hour^{-1}$ for PN solutions and 0.5 $mL \cdot hour^{-1}$ for lipids. Rates were increased gradually, so that a total fluid intake of 300 $mL \cdot kg^{-1} \cdot day^{-1}$ was achieved by 36 hours after surgery, and maintained thereafter.

Table 3.4 Estimated intravenous nutrient requirements for the piglets

Nutrient	Oral requirement[1] (4kg·day^{-1})	Oral requirement[2] (unit·kg^{-1}·day^{-1})	Bioavailability[3]	Estimated intravenous requirement[5] (unit·kg^{-1}·day^{-1})
Metabolizable energy, kcal	820	205	1.00	205
Amino acids, g	65.0	16.3	1.00	16.3
Indispensable amino acids, g	24	6.0	1.00	6.0
Dextrose, g	ND[4]	ND[4]	ND[4]	ND[4]
Sodium, mmol	27.4	6.85	0.90	6.14
Potassium, mmol	19.2	4.80	0.90	4.31
Calcium, mmol	56.1	14.0	0.50	7.0
Magnesium, mmol	4.1	1.03	0.57	0.59
Chloride, mmol	17.7	4.44	0.89	4.0
Phosphate, mmol	56.5	14.1	0.60	8.5
Zinc, μmol	382	96	0.91	87
Manganese, μmol	18.2	4.55	0.90	4.10
Copper, μmol	23.6	5.91	0.90	5.31
Chromium, μmol	ND[4]	ND[4]	ND[4]	ND
Selenium, μmol	1.0	0.25	0.91	0.23
Iodine, μmol	0.3	0.08	0.89	0.07
Iron, mg	25.0	6.3	0.14	0.9
Cobalamin, μg	5.0	1.25	1.00	1.25
Biotin, μg	20.0	5.0	1.00	5.0
Retinol, μg	189	47	1.00	47
Ergocalciferol, μg	1.4	0.34	1.00	0.34
α-Tocopherol, mg	2.7	0.67	1.00	0.67
Phylloquinone, μg	130	32.5	1.00	32.5
Ascorbic acid, mg	ND[4]	ND[4]	ND[4]	ND[4]
Thiamin, μg	380	95	1.00	95
Riboflavin, μg	1000	250	1.00	250
Pyridoxine, μg	500	125	1.00	125
Pantothenate, mg	3.0	0.75	1.00	0.75
Folate, μg	80	20	1.00	20
Niacinamide, mg	5.0	1.25	1.00	1.25
lipid g	32	8	ND[4]	ND[4]

1. From NRC (1998) nutrients requirements for swine weighing 3 to 5 kg.
2. Requirements above were assumed to represent a mean 4kg piglet, therefore the requirements were divided by 4 to estimate on a per kilogram basis.
3. The bioavailability adjusting for incomplete intestinal absorption (Wykes et al., 1993)
4. Not determined
5. The calculation in this table are: Oral requirement (4 kg) /4 = Oral requirement (unit·kg^{-1}·day^{-1}); Oral requirement (unit·kg^{-1}·day^{-1}) X Bioavailability = Estimated intravenous requirement (unit·kg^{-1}·day^{-1}).

Table 3.5 Formula of the PN solution

Product	Nutrient concentrations in the product		Volume added ($mL \cdot L^{-1}$)
10% Aminosyn PF[1]	Amino acid	100 $mg \cdot mL^{-1}$	610
70% Dextrose[2]	Dextrose	700 $mg \cdot mL^{-1}$	128
23.4% Sodium Chloride[3]	Na	4.00 $mmol \cdot mL^{-1}$	5.4
	Cl	4.00 $mmol \cdot mL^{-1}$	
Potassium Phosphate[3]	K	4.36 $mmol \cdot mL^{-1}$	5.4
	PO_4	3.00 $mmol \cdot mL^{-1}$	
Calcium Gluconate[4]	Ca	0.232 $mmol \cdot mL^{-1}$	75
50% Magnesium Sulfate[3]	Mg	4.15 $mmol \cdot mL^{-1}$	0.6
	SO_4	4.15 $mmol \cdot mL^{-1}$	
Iron Dextran Injection[3]	Fe	50 $mg \cdot mL^{-1}$	0.2
Vit B_{12} injection[1]	Vitamin B_{12}	0.1 $mg \cdot mL^{-1}$	0.1
M.V.I. Pediatric[3]	Vitamin A	460 $IU \cdot mL^{-1}$	4
	Vitamin C	16 $mg \cdot mL^{-1}$	
	Vitamin D	80 $IU \cdot mL^{-1}$	
	Thiamine	0.24 $mg \cdot mL^{-1}$	
	Riboflavin	0.28 $mg \cdot mL^{-1}$	
	Pyridoxine	0.2 $mg \cdot mL^{-1}$	
	Niacinamide	3.4 $mg \cdot mL^{-1}$	
	Panthenol	1 $mg \cdot mL^{-1}$	
	Vitamin E	1.4 $IU \cdot mL^{-1}$	
	Vitamin K_1	0.04 $mg \cdot mL^{-1}$	
	Biotin	4 $\mu g \cdot mL^{-1}$	
	Folic acid	28 $\mu g \cdot mL^{-1}$	
	Vitamin B_{12}	0.2 $\mu g \cdot mL^{-1}$	
Micro Plus 6 Pediatric[3]	Zinc	3 $mg \cdot mL^{-1}$	8.5
	Copper	0.4 $mg \cdot mL^{-1}$	
	Manganese	0.1 $mg \cdot mL^{-1}$	
	Chrome	4 $\mu g \cdot mL^{-1}$	
	Selenium	20 $\mu g \cdot mL^{-1}$	
	Iodine	60 $\mu g \cdot mL^{-1}$	
Water[2]	H_2O		163
Total			1000

1. Abbott Laboratories Canada: Montreal, Quebec, Canada.
2. Baxter Coroperation, Mississauga, Ontario, Canada.
3. Sabex Inc.: Boucherville, Quebec, Canada.
4. Pharmaceutical Partners of Canada Inc., Richmond Hill, Ontario, Canada.

Table 3.6 Estimated requirement achieved by the PN regimen

Nutrient	Estimated requirement[1] (unit·kg^{-1}·day^{-1})	Concentration in the PN solution (unit·L^{-1})	Intake during PN[2]	Requirement achieved by PN[3]	Energy %[4]
Metabolizable energy, kcal	205	550	214[5]	1.04	
Amino acids, g	16.3	61.0	17.1	1.05	32
Indispensable amino acids, g	6.0	30.0	8.4	1.41	
Dextrose, g	ND[6]	90.0	25.2	ND[6]	40
Sodium, mmol	6.14	21.46	6.01	0.98	
Potassium, mmol	4.31	23.36	6.54	1.52	
Calcium, mmol	7.0	17.4	4.9	0.70	
Magnesium, mmol	0.59	2.21	0.62	1.06	
Chloride, mmol	4.0	21.5	6.0	1.51	
Phosphate, mmol	8.5	16.1	4.5	0.53	
Zinc, µmol	87	393	110	1.27	
Manganese, µmol	4.10	15.61	4.37	1.07	
Copper, µmol	5.31	54.00	15.12	2.85	
Chromium, µmol	ND[6]	0.64	0.18	ND[6]	
Selenium, µmol	0.23	2.18	0.61	2.64	
Iodine, µmol	0.07	4.03	1.13	16.21	
Iron, mg	0.9	10.71	3.00	3.33	
Cobalamin, µg	1.25	11.57	3.24	2.59	
Biotin, µg	5.0	17.1	4.8	0.96	
Retinol, µg	47	600	168.0	3.55	
Ergocalciferol, µg	0.34	8.57	2.40	7.06	
α-Tocopherol, mg	0.67	6.00	1.68	2.51	
Phylloquinone, µg	32.5	171.4	48.0	1.48	
Ascorbic acid, mg	ND	68.57	19.20	ND	
Thiamin, µg	95	1029	288	3.03	
Riboflavin, µg	250	1200	336	1.34	
Pyridoxine, µg	125	857	240	1.92	
Pantothenate, mg	0.75	4.29	1.20	1.60	
Folate, µg	20	120	34	1.68	
Niacinamide, mg	1.25	14.57	4.08	3.26	
Lipids g	ND[6]	[7]	6.00	ND[6]	28

1. The estimated requirements were calculated in Table 3.6.
2. Intake during PN for the piglets were: the PN solution 280 mL·kg^{-1}·day^{-1}; lipids 20 mL·kg^{-1}·day^{-1}.
3. The calculation is: Concentration in the PN solution (unit·L^{-1}) X 280 mL /1000 = Intake during PN; Intake during PN / Estimated intravenous requirement = Requirement achieved by PN.
4. The calculation of energy were based on: Amino acids 4.0 kcal·g^{-1}; Dextrose 3.4 kcal·g^{-1}; Lipids 10.0 kcal·g^{-1}.
5. The energy of intake during PN is from the PN solution and the lipids. The calculation is :(550 kcal X 280 mL / 1000) + (6g X 10.0 kcal·g^{-1}) =214 kcal
6. Not determined.
7. The lipids were delivered separately from the PN solution.

3.2.3 Sample collection and analysis

Daily weight and daily nutrients intake were recorded. On day 0, 7, 14 and 21, 5 mL of venous blood was taken from the vascular access catheter into a metal free tube. The blood was centrifuged to get the serum. One mL serum was sent to the biochemistry lab in the Prairie Diagnostic Center in Western College of Veterinary Medicine, University of Saskatchewan, for serum alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), and bilirubin levels. Another 1 mL serum was sent to SRC for aluminum concentration. On day 21, the animals were euthanized. Urine, bile and liver samples were collected in metal free containers and sent to SRC for aluminum determinations. Liver sections were also obtained and fixed in 10% buffered formalin. The liver sample were processed with an automatic tissue processor, and stained with haematoxylin and eosin. The liver slides were sent for histological evaluation to detect canalicular plugging, periportal inflammation and fibrosis. The aluminum levels in the commercial products used for PN solutions were determined. The aluminum concentrations in 5 random samples of PN solutions used for the PN group were measured.

ALP, GGT and bilirubin were assayed on a Beckman-Coulter LX-20 by standard enzymatic method (Fody E.P., 2000). PN solutions, serum, bile, urine and hepatic aluminum were determined at SRC by inductively coupled plasma atomic emission spectrometry (ICP-AES). The pathology specimens were processed at the Surgical Research Laboratory, University of Saskatchewan, and evaluated by the veterinary pathologist at the Prairie Diagnostic Services Center, Western College of Veterinary Medicine, University of Saskatchewan.

3.2.4 Statistical methods

Descriptive statistics was performed. Results are expressed as mean ± standard deviation (SD). One-way ANOVA and Post Hoc (Student-Newman-Keuls) were used to compare the variables in different groups. Values were considered significant at $p<0.05$.

3.2.5 Results

The aluminum content in saline used for the Control group and for flushing all central venous access catheters was less than 1 $\mu g \cdot L^{-1}$. The aluminum levels in the solutions formulated for the Low Al group (20 $\mu g \cdot mL^{-1}$) and the High Al group (1500 $\mu g \cdot mL^{-1}$) were confirmed by the ICP-AES testing in SRC. The aluminum content of all the solutions used in the PN group was measured (Table 3.7). Based on the formulation of PN solution, the aluminum content of PN solution for the piglet was calculated at 350.1 $\mu g \cdot L^{-1}$. The mean aluminum content of 5 random PN solutions was 332.4±7.7 $\mu g \cdot L^{-1}$ (322 to 342 $\mu g \cdot L^{-1}$). Based on the amount of the fluids delivered by PN in the piglets, the mean aluminum intake for PN group was estimated at 37.8±14.3 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (Table 3.8).

There was no significant difference in growth patterns among the control group, the Low Al group and the High Al group (Table 3.9). At day 14 and day 21, the bodyweights of the PN group was significantly lower than other three groups (Figure.3.7). Serum ALP and GGT levels was shown in Table 3.10. Serum ALP was significantly elevated in the Low Al and High Al group at day 7. There was no significant difference among four groups in mean serum ALP at day 0, day 14, and

Table 3.7 Aluminum content in PN solutions

Product[1]	Aluminum ($\mu g \cdot L^{-1}$)	PN solution ($mL \cdot L^{-1}$)	Aluminum[4] (μg)
10% aminosyn PF	<50[2]	610	15.3
70% Dextrose	<50[2]	128	3.2
23.4% Sodium Chloride	<100[2]	5.4	0.3
Potassium Phosphate	6300	5.4	34.0
Calcium Gluconate	3900	75	292.5
50% Magnesium Sulfate	<300[2]	0.6	0.1
Iron Dextran	3200	0.2	0.6
Micro Plus 6 Pediatric	290	8.5	2.5
M.V.I. Pediatric	375	4	1.5
VitB12	<50[2]	0.1	0.0
water	0.7	163	0.1
30% Intralipid[3]	60		
Total		1000	350.1

1. The nutrient contents and manufactures of the products were listed in Table 3.3.
2. When the aluminum levels under the detection limits, half of the value was used in the calculation.
3. One mL contains 30 mg lipids. Fresenius Kabi AB, Uppsala, Sweden.
4. The calculation of aluminum is: Aluminum ($\mu g \cdot L^{-1}$) X PN solution ($mL \cdot L^{-1}$) / 1000 = Aluminum (μg)

Table 3.8 An example of the calculation of aluminum intake from PN therapy in the PN group piglets[1]

	Aluminum content ($\mu g \cdot L^{-1}$)	Intake from PN therapy ($mL \cdot kg^{-1} \cdot day^{-1}$)	Aluminum intake[6] ($\mu g \cdot kg^{-1} \cdot day^{-1}$)
PN solutions[2]	332.4[3]	121	40.2
30% intralipids[4]	60[5]	14	0.8
Total			41.0

1. The intake from PN therapy is from the piglet (subject 31) in the PN group at the day 5 of the experiment.
2. The formula of PN solutions for the piglets was listed in Table 3.7.
3. The aluminum amount in the solution was the mean of 5 random samples determined at Saskatchewan Research Council (SRC), Saskatoon.
4. One mL contains 30 mg lipids. Fresenius Kabi AB, Uppsala, Sweden.
5. The amount of aluminum was determined at Saskatchewan Research Council (SRC), Saskatoon.
6. The calculation of aluminum intake is: Aluminum content ($\mu g \cdot L^{-1}$) X Intake from PN therapy ($mL \cdot kg^{-1} \cdot day^{-1}$) / 1000 = Aluminum intake ($\mu g \cdot kg^{-1} \cdot day^{-1}$)

Table. 3.9 The mean body weight of piglets on Day 0, 7, 14 and 21.

Group	Weight (g)			
	Day 0	Day 7	Day 14	Day 21
Control (n=5)	2220±259	4040±476	5960±442[a]	8300±774[a]
Low Al (n=7)	1857±544	3836±1098	5843±1300[a]	7585±1570[a]
High Al (n=6)	2275±555	4058±498	5975±1145[a]	8542±1700[a]
PN (n=6)	2383±634	3417±694	4625±539[b]	5463±562[1,b]

Data was expressed as mean±SD. One-way ANOVA and Post Hoc test (Student-Newman-Keuls) was used to compare means. Means with different superscripts were significantly different ($p<0.05$).
1. n=4 at day 21 in PN group.

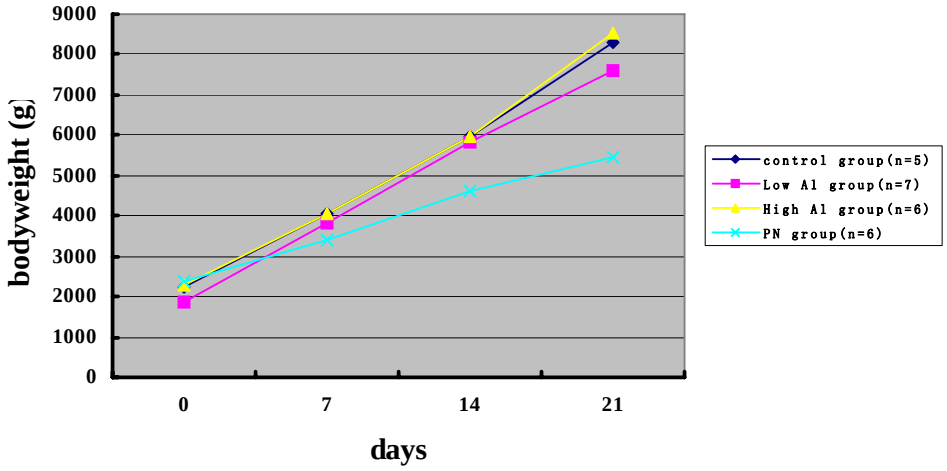


Figure 3.7 The pattern of growth in all piglets

The symbols (■, ♦,▲, and ⋆) represent means. n=4 at day 21 in PN group. The mean body weights in PN group piglets at day 14 and day 21 were significant lower than other three groups.

Table 3.10 The mean serum ALP, GGT and total bilirubin of the piglets during the experimental period

Group	ALP ($U \cdot L^{-1}$)				GGT ($U \cdot L^{-1}$)				Total bilirubin ($\mu mol \cdot L^{-1}$)			
	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21
Control (n=5)	1282±349	1138±564[a]	749±266	529±87	47±10	60±21	55±20[a]	60±28[a]	1.0±0.7	1.8±1.1[a]	2.2±1.3[a]	1.6±0.9[a]
Low Al (n=7)	1654±525	2406±778[b]	901±410	403±200	38±12	42±14	31±6[a]	29±10[a]	1.7±0.9	3.1±1.7[a]	2.7±1.5[a]	3.0±1.4[a]
High Al (n=6)	1682±726	2257±421[b]	1458±572	799±389	48±21	58±23	58±35[a]	47±33[a]	1.3±0.8	5.7±1.4[b]	6.8±1.3[b]	7.2±1.6[b]
PN (n=6)	3222±2782	1239±753[a]	989±522	802±299[1]	37±13	43±18	147±124[b]	130±89[1,b]	2.0±0.6	13.5±17.4[c]	62.8±38.9[c]	109.5±57.3[1,c]

Data was expressed as mean±SD. One-way ANOVA and Post Hoc test (Student-Newman-Keuls) was used to compare means. Means with different superscripts (a, b, c) were significantly different ($p<0.05$) when compare to each other. The normal value for serum ALP is 110-340 $U \cdot L^{-1}$, for serum GGT is 0-25 $U \cdot L^{-1}$, for serum total bilirubin is 0-4 $\mu mol \cdot L^{-1}$.

1. PN group only had 4 piglets at day21.

day 21. A significant elevation in mean serum GGT showed in the PN group at day 14 and day 21 (Table 3.10).

The mean serum total bilirubin was significantly increased in the High Al and PN group at day 7, 14, and 21 (Table 3.10). Note that 2 of 7 (28.6%) piglets in the Low Al group had serum bilirubin over 4 $\mu mol \cdot L^{-1}$ (normal value). The mean serum aluminum concentration was significantly increased in the High Al group at day 7, 14 and 21 (Table 3.11). For other three groups, there were no significant differences for mean serum aluminum concentration during the experimental period. Mean liver aluminum concentrations at the end of the study were significantly elevated in all three experimental groups when compared to the Control group and they were dose dependant (Table 3.11). Mean urine aluminum concentrations at the completion of the experiment were significantly increased in the PN group and the High Al group, but not in the Low Al group. Mean bile Al concentrations were elevated only in the Low Al group at the end of the study. However, it should be noted that for 3 of 6 piglets in the PN group and 1 of 6 piglets in the High Al group, there was no bile can be obtained at the end of study.

Histological evidence of mild to moderate cholestasis was observed in 1 control subject, 2 Low Al subjects, 1 High Al subject and all 6 subjects in the PN group (Table 3.12).

Table 3.11 Serum, liver, urine and bile aluminum concentrations in the piglets

Group	Serum aluminum ($\mu g \cdot L^{-1}$)				Liver aluminum ($\mu g \cdot g^{-1}$)	Urine aluminum ($\mu g \cdot L^{-1}$)	Bile aluminum ($\mu g \cdot L^{-1}$)
	Day 0	Day 7	Day 14	Day 21			
Control (n=5)	46±32	20±11[a]	33±37[a]	38±32[a]	0.05±0.06[a]	19±7[a]	16±2
Low Al (n=7)	53±50	239±324[a]	126±97[a]	79±74[a]	0.87±0.60[b]	84±93[a]	64±46
High Al (n=6)	27±16	1187±473[b]	1036±753[b]	880±807[b]	71.33±7.78[c]	1521±1937[c]	289±385[2]
PN (n=6)	14±4	45±34[a]	45±16[a]	37±22[1,a]	0.90±0.37[b]	259±198[b]	17±10[3]

Data was expressed as mean±SD. When the concentration was under the detected limitation, half of the value was used in the calculation. One-way ANOVA and Post Hoc test (Student-Newman-Keuls) was used to compare means. Means with different superscripts (a, b, c) were significantly different ($p<0.05$) when compared to each other.

1. n=4, PN group only had 4 piglets at day21.
2. n=5, for one piglet in High Al group there was no bile could be obtained at the end of the study.
3. n=3, for three piglets in PN group there were no bile could be obtained at the end of the study.

Table 3.12 The histological changes in the piglets

Subject number	Normal liver	Mild hepatitis	Minimal hepatic fibrosis	Mild extramedullary haematopiesis	Mild to moderate cholestasis
Control					
11			X		
14		X			
16					X
21		X			
25	X				
Subtotal (n=5)	n=1	n=2	n=1	n=0	n=1
Low Al					
7		X			
8		X			
9		X		X	
10				X	
20		X			X
24	X				
26		X			X
Subtotal (n=7)	n=1	n=5	n=0	n=2	n=2
High Al					
18		X			
19	X				
27		X		X	
28		X	X		
29				X	
30		X		X	X
Subtotal (n=6)	n=1	n=4	n=1	n=3	n=1
PN					
31					X
32					X
33					X
34					X
35				X	X
36				X	X
Subtotal (n=6)	n=0	n=0	n=0	n=2	n=6
Total (n=24)	n=3	n=11	n=2	n=7	n=10

3.2.6 Discussion

A neonatal piglet model was developed to investigate aluminum in PNAC. The growth pattern was an important consideration in model development. Despite the different doses of intravenous aluminum, the piglets in the control, Low Al and High Al group had a similar growth pattern. The body weight of piglets in the PN group was significant lower than the other three groups after day 7. It is also significant that all groups other than the PN group had free access to food. A slow growth pattern was also seen in the infants who were on PN therapy, especially for a long term (Putet, 1993).

Intravenous aluminum used in the Low Al group represents the current aluminum contaminant level of PN solutions in the hospitals. The high dose used in the High Al group was 1500 $\mu g \cdot kg^{-1} \cdot day^{-1}$, which was the dose used in Klein's study (Klein, et al., 1987). The differences were that the animals in Klein's study were 6-week old piglets and the experimental time was 50 days (Klein et al., 1987). This High Al group was an external control in the study. The significant elevated serum aluminum levels and elevated total bilirubin levels in this group verified Klein's work and demonstrated that the piglet model was effective to study the role of aluminum in cholestasis.

A significant elevation in serum total bilirubin indicating the development of cholestasis was found in the High Al group and the PN group, but not in the Low Al group. This suggested that high dose parenteral aluminum might play a role in the development of cholestasis. The data confirmed previous animal studies that

aluminum could cause hepatic dysfunction. PNAC was developed in the PN group. However, the failure to demonstrate this change in the Low Al group would make caution in extrapolating the role of aluminum in the development of PNAC into clinical situations. The elevated serum bilirubin in the PN group agreed that other factors such as PN therapy or lack of enteral feeding might interplay with parenteral aluminum in the development of cholestasis. Serum total bilirubin kept climbing during the experiment in the PN group. Thus, a relationship between the time on PN and the degree of cholestasis was implied in the PN group. Although there was no significant difference in serum total bilirubin found in the Low Al group, two of seven piglets (piglet 7 and piglet 8) had an abnormal value (see Table 6.3 in Appendix D). This indicated that the hepatic dysfunction might be present in these two piglets.

Serum ALP showed a mixed result in the piglets. Serum GGT only significantly elevated on day 14 and day 21 in the PN group. The data from both piglets and infants suggested that serum ALP and serum GGT were not good markers of cholestasis. There are other liver enzymes such as serum alanine aminotransaminase (ALT, or SGPT) and aspartate aminotransaminase (AST, or SGOT), which could be used as the markers of liver cell injury. However, the common causes of the elevations in ALT and AST are fatty liver diseases seen most often in those with obestity, diabetes or elevated blood lipids, but not in cholestasis (Fody, 2000).

Serum aluminum level was significantly elevated only in the High Al group.

Note that high standard deviation was found in serum aluminum concentration both in the piglets and the infants. Different abilities to excrete the aluminum into urine or bile may affect the serum aluminum levels. Moreover, the rate of aluminum deposition in the organs may differ among the individual subjects. Therefore, even under the same contaminated aluminum level, serum aluminum may not reflect the parenteral aluminum intake. High variable bile and urine aluminum concentrations in the piglets may reflect differing excretion capabilities. And without the correlation with serum total bilirubin in piglets, serum aluminum was not a good indicator of cholestasis. Similar result was shown in our infant study. Previous human studies of infants on long term PN therapy suggested an elevated serum aluminum concentration (Moreno et al., 1994; Koo et al., 1986). Thus, further research is required to demonstrate if the trend of serum aluminum would go up again with the continuing unsafe aluminum loading.

Liver aluminum concentration was significantly ($p<0.05$) elevated in all the groups compared to the control group and the relationship was dose dependant. Hepatic aluminum accumulation may in some way cause injury of the liver. A recent study has shown a chronic aluminum exposure impaired the biliary secretory function and caused cholestasis in rats (Gonzalez et al., 2004). The concentrations of aluminum deposited in the liver of the experimental group of piglets, especially the Low Al group and the PN group were lower than those found in chronically loaded infants receiving aluminum contaminated PN solutions (Klein et al., 1984). The explanation could be: 1. The excretion of aluminum in these piglets was higher

than the chronically loaded infants; 2. The rate of aluminum hepatic deposition was different between these piglets and the infants; 3. The experimental time was shorter than the duration of PN therapy in the infants. Therefore, extending the experiment time may increase the accumulation of aluminum in the liver, and further liver damage may show in the Low Al group piglets. More information about the aluminum excretion ability and hepatic deposition in both piglets and infants would be helpful.

The failure to detect consistent histological signs of cholestasis in the High Al group suggested that the elevation of serum total bilirubin may precede or be independent of the appearance of other hepatic pathology as has been suggested to occur in cholestasis. No positive hepatic histology findings have also been shown in previous animal models (Klein et al., 1987; Klein et al., 1988). The histological findings of cholestasis in the PN group confirmed that more severe cholestasis developed in these piglets.

Although we had the aluminum content of urine and bile evaluated at the end of the study, more information was required to determine the excretion ability of the piglets. Small sample size was another limitation of the study. A longer experiment time may help to see more significant results, especially in the Low Al group.

3.2.7 Conclusion

A neonatal piglet model was successfully developed to investigate the role of aluminum in PNAC. Hepatic aluminum accumulation was found and it was dose

dependent. High dose of intravenous aluminum or PN therapy with contaminated aluminum can cause cholestasis in the piglets. However parenteral aluminum alone, at doses comparable to contamination level of PN therapy in the infants did not. Serum aluminum levels did not directly correlate with the development of cholestasis. This may due to the differing excretion capabilities of the piglets. The current aluminum contaminated level in PN solutions may interact with other potential factors such as PN therapy or lack of enteral feeding and play a role in PNAC.

4. SUMMARY AND FUTURE DIRECTIONS

PNAC is a common complication of PN therapy in infants. The etiology of PNAC is still unclear and is likely to be multifactorial. The potential factors which have been previously implicated include prematurity, low birth weight, lack of enteral feeding, duration of PN and sepsis. Also, the components of PN solution including the nutrients and the contaminants, such as aluminum, have been considered as potentially causative factors. Aluminum contaminated in PN solutions bypasses the gastrointestinal barrier and directly enters the circulation, leading to accumulation in the liver. However, the exact role of aluminum in PNAC required further research.

We investigated the role of aluminum in human infants with gastrointestinal failure who required PN by monitoring the relationship between serum aluminum concentration and serum direct bilirubin concentration. Demographical information was collected including: birth weight, gestation age, and diagnosis. Serum aluminum and bilirubin (direct and indirect) concentrations were determined on days 0, 7, 14, and 21. PNAC was diagnosed in infants with a serum direct bilirubin $\geq$34 $\mu mol \cdot L^{-1}$. Aluminum concentrations were also measured in representative samples of PN solutions. This was used to estimate the daily intake of aluminum per kilogram of body weight from PN. By the end of the study (21 days), 5 of 16 (31.3%) infants developed PNAC. We found that serum aluminum concentrations

peaked at day 7 of PN therapy and declined thereafter. There was no significant difference in serum aluminum concentrations between PNAC and non-PNAC infants. Furthermore, a significant correlation between the serum direct bilirubin and serum aluminum concentrations was not observed. There were no significant differences between the PNAC and non-PNAC group in birth weight, gestation age and duration of PN therapy.

Without the significant findings between serum aluminum and the development of PNAC in infants, the role of aluminum in PNAC is blurred. The comparison of different doses of parenteral intake of aluminum will give more details of the role of aluminum in this disease. The evaluation of not only serum aluminum concentration, but also the aluminum levels in liver, urine and bile will help to understand the whole picture of aluminum in this disease. For practical and ethical reasons, this type of study would be best conducted in an animal model. We developed a piglet model to further study the relationship between different doses of intravenous aluminum or PN therapy and the development of cholestasis. Twenty-four piglets, 2 to 4 days old, underwent insertion of a silastic central venous access catheter and received either daily intravenous (i.v.) aluminum injections or PN solutions for 3 weeks. The groups were: 1. Control group (n=5); 2. Low Al (aluminum) group (n=7), intravenous (iv) injection with aluminum dose at 20 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (the approximate aluminum level in PN solutions for the infants); 3. High Al (aluminum) group (n=6), iv with aluminum dose at 1500 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (the level caused cholestasis in pigs by a previous investigator); 4. PN (parenteral

nutrition) group (n=6), PN solutions with a mean aluminum dose at 37.8±14.3 $\mu g \cdot kg^{-1} \cdot day^{-1}$. Serum bilirubin and aluminum was measured on day 0, 7, 14, 21. At the completion of the study, urine, bile, and liver aluminum concentration were measured. The piglet study demonstrated that aluminum concentrations comparable to contamination levels in current PN solutions (20 $\mu g \cdot kg^{-1} \cdot day^{-1}$) were unable to produce cholestasis in piglets by 21 days. However, cholestasis did develop in the piglets with the high dose aluminum (1500 $\mu g \cdot kg^{-1} \cdot day^{-1}$) treatment and in the piglets with PN therapy (the aluminum dose was 37.8±14.3 $\mu g \cdot kg^{-1} \cdot day^{-1}$). Hepatic aluminum accumulation was found in all experimental piglets and it was dose dependent. The result of this study showed that the hepatic accumulation of aluminum occured even in the low level of parenteral intake of aluminum (20 $\mu g \cdot kg^{-1} \cdot day^{-1}$) by 21 days.

The components of PN solutions in both infant and piglet study were the same as the components used in the local hospital. The mean aluminum parenteral intake in the infant study was 21.6 $\mu g \cdot kg^{-1} \cdot day^{-1}$. Five of sixteen (31.3%) infants developed cholestasis by 21 days. In the piglet study, the low aluminum group with the similar aluminum intake (20 $\mu g \cdot kg^{-1} \cdot day^{-1}$) did not develop cholestasis. However, cholestasis developed in the high aluminum group (1500 $\mu g \cdot kg^{-1} \cdot day^{-1}$) and PN group (aluminum intake was 37.8±14.3 $\mu g \cdot kg^{-1} \cdot day^{-1}$) by 21 days. The difference of aluminum intake between the infants and the PN group piglets was due to the different requirements of infants and piglets. The nutrients requirements of piglets are much higher than the infants. It would be interesting to see: 1. If we

extend the experimental time, would the piglets in the low aluminum group develop cholestasis? 2. If the 37.8 $\mu g \cdot kg^{-1} \cdot day^{-1}$ aluminum level was used in the piglets, would that result in cholestasis? The aluminum concentration in PN solutions may vary based on the different products and manufactures. In the United States, the legislation was made to limit the amount of aluminum in the PN solutions. The aluminum levels in the large volume solutions such as amino acids, dextrose, and lipids must less than 25 $\mu g \cdot L^{-1}$. However, there was no limitation for the small volume solutions such as calcium gluconate, which was the major contributor of aluminum contamination. The aluminum in the component of calcium gluconate accounted for more than 80% of total aluminum in both infant and piglet PN solutions (83% in the piglet PN solution and 85% in the infant PN solution, see Table 3.2 and Table 3.7). Since aluminum is hard to remove by the manufactures, minimizing the amount of calcium gluconate in the PN solution would significantly reduce the aluminum contaminate level. There is still not enough data in infants to define a safe level. Additional human studies need to be conducted to more fully explore this issue.

The wide variation of serum aluminum levels was found in both infant and piglet studies. Since there was similar parenteral intake of aluminum in both studies, the variation in serum aluminum levels may be due to different abilities to excrete aluminum. Moreover, the rate of aluminum deposition in the organs may differ among the individual subjects. The high variable bile and urine aluminum concentrations in the piglets reflected the differing excretion capabilities. However,

the data on the individual physiology in the handling and excretion of aluminum was not completed. The total amount of aluminum excreted in urine or in bile was not assessed. The limited data from previous studies on this issue requires further studies.

The diagnosis of cholestasis is made on the basis of the elevated serum direct bilirubin. In the infant study, PNAC was diagnosed when the serum direct bilirubin was greater than 34 $\mu mol \cdot L^{-1}$. The diagnosis standard was adapted from the previous infant studies. The normal value of serum direct bilirubin in infants is 0 to 5 $\mu mol \cdot L^{-1}$. We found that 13 of 16 infants had a direct hyperbilirubinemia (serum direct bilirubin greater than 5 $\mu mol \cdot L^{-1}$). Only 5 infants reached the diagnostic cut line (serum direct bilirubin greater than 34 $\mu mol \cdot L^{-1}$) by the end of the study. Direct hyperbilirubinemia is abnormal and is seen in association with PN therapy. Setting the level of 34 $\mu mol \cdot L^{-1}$ as the diagnosis of PNAC is arbitrary. Selecting a lower value increases the incidence but also increases the risk of false PNAC. However, the current level is much higher than the normal level. Further more, the evidence to support the current diagnostic standard which has been used by most of the investigators can hardly be found.

The aim of the animal model is to provide a comprehensive and in-depth picture of the role of aluminum in PNAC. Piglets were selected because of the similarity of liver function and bile secretion between human infants and neonatal piglets. The piglets enrolled in our study were healthy and the infants were sick. The mean weight gained in the piglets by 21 days was 252 ± 31 $g \cdot day^{-1}$, which was

much higher than the mean weight gained in the infants (16±22 $g \cdot day^{-1}$) during the study. The different grow rate may affect the rate of maturity of liver and kidney in these subjects. Limited data can be found comparing liver and kidney functions between infants and piglets in the first 3 weeks of life. The neonatal piglet model also does not represent premature human infants in physiology. Development of a preterm piglet model may help to better understand PNAC in premature infants.

PNAC developed within 21 days in 5 infants. In the piglet study, two group piglets (High aluminum and PN group) developed cholestasis, but not in the piglets in the Low aluminum group. A statistically significant correlation between serum aluminum concentration and serum bilirubin was not observed in both infant and piglet studies. The impact of aluminum in cholestasis may depend on the amount of parenteral intake of aluminum and the presence of other potential factors. The interaction between aluminum and other potential factors requires further research, especially at the low aluminum level.

Since the accumulation of aluminum in the liver was shown in the piglet study and it was dose dependent, the relationship between hepatic aluminum deposit and the transport system of hepatocyte would further clarify the mechanism of aluminum in PNAC. A recent study showed that chronic exposure to aluminum impaired the hepatic handling of organic anions by decreasing both sinusoidal uptake and canalicular excretion in rats (Zambrano et al., 2004). Additional studies are needed to confirm and clarify the exact mechanism through which aluminum wields these harmful effects. The recent opening of the Canadian Light Source on the

University of Saskatchewan campus may help us to know more details about the microstructures of hepatic tissues of our animal model. This kind of microscopic evaluation has not been reported.

In conclusion, our results showed that the role of aluminum in cholestasis may depend on the amount of parenteral intake of aluminum. Other factors such as the individual ability to excrete aluminum, and the lack of enteral nutrition, may play complimentary roles. The result of infant study has significantly contributed to the previously limited data of aluminum in infants with PNAC. One of the limitations of the infant study is the sample size. Expanding the sample size and extending the experimental time may show some statistical significance. The individual ability to excrete aluminum will be assessed in both infant and piglet study in the future. Different doses of aluminum other than 20 $\mu g \cdot kg^{-1} \cdot day^{-1}$, such as 40 $\mu g \cdot kg^{-1} \cdot day^{-1}$, will be used in the developed piglet model to further study the role of aluminum in PNAC. Other types of piglet model such as a preterm neonatal piglet model will be developed based on the experiences of this model. The better understanding of the process involved in the development of PNAC would significantly contribute to the interventions for reducing the incidence and the severity of this disease in infants.

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6. APPENDICES

Appendix A

Ethics Certificates

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	BMC#
G.G. Miller	Surgery	2000-96

INSTITUTION(S) WHERE RESEARCH WILL BE CONDUCTED (STUDY SITE)

Royal University Hospital
103 Hospital Dr
Saskatoon SK S7N 0W8

SPONSORING AGENCIES

HSURC

TITLE:

Aluminum Toxicity and Parenteral Nutrition Associated Cholestasis in Neonates

APPROVAL DATE	EXPIRY DATE
April 14, 2000	May 1, 2001

CERTIFICATION

The University Advisory Committee on Ethics in Human Experimentation (UACEHE) has reviewed the above-named research project including the protocol and consent form, if applicable. The proposal was found to be acceptable on ethical grounds. The principal investigator has the responsibility of ensuring that the authorized research is carried out according to governing law. This Certificate of Approval is valid for the above time period provided there is no change in experimental procedures.

ONGOING REVIEW REQUIREMENT(S)

The UACEHE will require the submission of an annual status report prior to the expiry date of May 1, 2001.

APPROVED.

D.W. Quest, Chair
University Advisory Committee on
Ethics in Human Experimentation

Please send all correspondence to:

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CONSENT FORM

Aluminum Toxicity and Parenteral Nutrition Associated Cholestasis in Neonates

Principal Investigator: Grant G. Miller, MD, Dept. of Surgery, University of Saskatchewan, 966-8141

Co-investigators:
G.A. Zello, PhD, Division of Nutrition and Dietetics, University of Saskatchewan, 966-5825
N.A. Wonko, MD, Dept. of Pediatrics, University of Saskatchewan, 966-8118

Background

As part of your child's care his/her doctor has decided to give nutrition (food) by an intravenous fluid referred to as TPN. In some cases when TPN is used for many weeks the child may develop jaundice (cholestasis) due to decreased bile flow from the liver. This may cause damage to the liver that may be mild or in some cases severe. We do not know the cause for this. Small amounts of aluminum are present in the TPN solutions. It is not an essential component of the TPN. It is possible that the aluminum builds up in the liver and causes the jaundice.

Purpose of this Study

The purpose of this study is to determine if infants receiving TPN accumulate aluminum in their bodies and if this is associated with the development of cholestasis (jaundice).

Study Procedure

Before your child is started on TPN we will test his/her blood for aluminum and jaundice. This will require taking 2 milliliters of blood. These blood tests will be repeated once per week for 3 weeks.

Possible Benefits

If we can determine that aluminum does cause cholestasis then altering the TPN solutions to reduce or eliminate the aluminum may prevent this complication.

Risks, Side Effects and Discomfort

The blood will be drawn in the standard fashion similar to what you may have experienced if you have ever had blood tests. There will be the expected pain of inserting a needle to withdraw the blood. In some cases there may be some bruising of the skin at the needle site. There is a risk of injuring surrounding tissues when the needle is inserted. This is a rare complication. Trained personnel such as a phlebotomist, nurse or doctor will draw the blood. Other unforeseen complications could occur.

Withdrawal from the Study

You may withdraw your child from participation in this study at any time. Withdrawal will in no way affect your child's access to health care or other services.

Confidentiality

All information from this study will be confidential. Information from this study may be published in medical journals, thesis or presented at academic or medical meetings. Individual patients will not be identifiable in these reports.

Questions

If you have any questions regarding this research project please contact Dr. G. Miller at (306) 966-8141.

New Information

We will advise you of any new information that arises that may have a bearing on your decision to continue enrollment in this study.

Consent

The study and the contents of this consent form have been explained to me. I understand that participation in this study is entirely voluntary and that I may refuse to participate or I may withdraw my child from the study at any time without any consequences to his/her continuing care.

I have received a copy of this consent for my own records.

I consent to the participation of:

---------- in this study.
(Child's name)

---------- Date: ----------
(Signature of parent or legal guardian)

---------- Date: ----------
(Signature of witness)

---------- Date: ----------
(Signature of researcher or designate)

CERTIFICATION OF ANIMAL CARE

Principal Investigator: Dr. G. Miller

Department: Surgery

University of Saskatchewan, Saskatoon, SK S7N 5C4

Title of Grant Application: The role of aluminum in parental nutrition associated cholestasis in neonates

UCACS Protocol Number: 20000035

The local Animal Care Committee, having received the protocol relevant to a The Hospital for Sick Children Foundation grant application to support the above-named project, on matters relating to animal care and treatment, will examine the experimental procedures proposed. It certifies with the applicant that the care and treatment of animals used will be in accordance with the principles published in the Canadian Council on Animal Care's "Guide to the Care and Use of Experimental Animals."

Further, all matters arising from this proposal that relate to animal care and treatment, and all experimental procedures proposed for use with animals will be reviewed and approved by the Animal Care Committee before they are initiated or undertaken by the applicant. This review process will be ongoing on a regular basis during the entire period that the research is being undertaken.

Signatures:

Applicant

Oct 20/00
Date

Irene Tollefsen for;
Chairman, Dr. Chuck Rhodes
Local Animal Care Committee

October 17, 2000
Date

The University certifies that it will monitor adherence to these guidelines.

Office of Research Services

Oct 27, 2000
Date

University Committee on Animal Care and Supply, c/o Animal Resources Centre, University of Saskatchewan
120 Maintenance Road, Saskatoon SK S7N 5C4 Canada Telephone: (306) 966-4126 Facsimile: (306) 966-8850

Appendix B

Study Protocols

PROTOCOL
THE ROLE OF ALUMINUM IN THE PARENTERAL NUTRITION ASSOCIATED CHOLESTASIS IN INFANTS

Investigators: Dr. G. Miller, Dr. G. Zello
Graduate Student: Mei Li
Contact: Mei Li at 9665831

Parenteral nutrition associated cholestasis (PNAC) is a common complication of parenteral nutrition (PN) in infants. It has been demonstrated in experimental models and early human studies that the contaminant aluminum in the PN solutions is related to the development of cholestasis. Our objective is to measure the relationship between the development of cholestasis in infants receiving PN and the concentration of aluminum in these infants blood.

Inclusion criteria: Infant, age <365 days
Gastrointestinal failure: gastroschesis, intestinal atresia, omphalocele, necrotizing eterocolitis, intestinal aganglionosis

Exclusion criteria: 1. Congential hepatic or biliary disorder such as: extrinsic obstruction of the biliary system, hepatic neoplasm, inspissated bile syndrome, spontaneous perforation of the common bile duct, infantile obstructive cholangiopathy (neonatal hepatitis, biliary atresia, biliary hypoplasia, choledochal cyst, Alagille's syndrome)
2. Major cardiac anomaly
3. Other congenital or acquired disorders which may cause hepatic disease such as: hepatitis, syphilis, rubella, toxoplasmosis, metabolic disorders (i.e., galactosaemia, fructosaemia, alpha-1-antitrypsin deficiency, cystic fibrosis) and endocrine disorders (i.e., hypothyroidism, hypopituitarism, hypoadrenalism)

Methods:

1. Identify potential subjects
2. Contact Mei Li at 9665831, College of Pharmacy and Nutrition, U of S
3. Obtain informed consent
4. Draw baseline bloodwork: 2mL blood in the metal free tube (blue cap, supplied by Mei Li in the research binder on the shelf in NICU).
5. Obtain weekly bloodwork for 3 weeks: same as baseline
6. Fill the reminder form of bloodwork.

BLOOD WORK REMINDER FORM

The Role of Aluminum in Parenteral Nutrition Associated Cholestasis in Infants

Investigators: Dr. G. Miller, Dr. G. Zello

Graduate Student and Contact Person: Mei Li (966 5831)

Patient's name:

PIN:

Bloodwork: Please check off when blood work is done.

	Day 0	Day 7	Day 14	Day 21
Total bilirubin				
Direct / indirect				
Alkaline Phosphatase (ALP)				
Gamma glutamyl transferase (GGT)				
Aluminum				

- Use the Navy tube supplied by the investigators.
- Label "Aluminum blood work" with EC# 2000-96.
- Send all the blood to the lab in RUH.

PROTOCOL
THE ROLE OF ALUMINUM IN THE PARENTERAL NUTRITION ASSOCIATED CHOLESTASIS IN PIGLETS

Investigators: Dr. G. Miller, Dr. G. Zello
Graduate Student: Mei Li
Contact: Mei Li at 9665831

A week before

- Set up the starting date, check the availabilities of the labs needed in the study and inform all the staffs involed
  Dr. G. Miller, Michele, Dawna
  Prairie Swine Center, surgery lab, animal housing, sterile lab

Day 0 (Monday)

- (8:00-9:00) Piglets (2-3 days old) are usually arrive on Monday morning and reception weights are obtained (Health science building).
- (9:00-12:00) PN solutions and injectable aluminum solution preparation (sterile lab).
- (13:00) Surgery, catheter is placed and baseline blood work is drawn.
- (20:00) Check the piglets to see if it is awake after the surgery.

Day 1 (Tuesday) to Day 21 (Monday)

- (8:00-10:00) Weight the piglets, record intake, clean up the cage and floor, give daily aluminum injection. Draw blood every Monday (on day 7,14, and 21).
- (10:00-12:00) PN solutions preparation
- (12:00) Change the PN bags, adjust the pump rates to achieved the requirement
- Day 21, animals are euthanized, urine, bile, liver and kidney are obtained. Liver is harvested, weighted, section taken for staining, another section frozen for aluminum determination, the left section is frozen for storage.
- Send serum sample to the Western College of Veterinary Medicine for liver function tests
- Send serum, urine, bile, liver, PN solutions, injectable aluminum solutions for aluminum determination at SRC. (Call Brenda in advance)
- Once the slides of the liver are prepared, take them to Prairie Diagnostics Center for pathology reading in the Western College of Veterinary Medicine.

PROTOCOL FOR PN SOLUTIONS PREPARATION

1. Laminar airflow hood (LAH) should be started and run for 30 minutes prior to cleaning and compounding in the hood.
2. Clean LAH first with the germicide cleaning solution
3. Clean LAH with 70% alcohol solution.
4. All products introduced into the LAH if not removed from an over-wrap should be sprayed with 70% alcohol prior to placing in the LAH.
5. Over-wrap should be opened at the edge of the LAH to minimized particulate matter in the critical workspace area.
6. Clamp all tube feeds into the PN bag.
7. Spike 1 vented lead into the glass amino acid bottle.
8. Spike 1 vented lead into the dextrose bag.
9. Hang the bottle and the dextrose bag on the IV bar in the LAH and tare the PN bag on the scale.
10. Release clamp on the dextrose lead and by gravity drain into the PN bag to the desired weight. Re-clamp lead.
11. Tare scale. Release clamp on the amino acid lead and by gravity drain into the PN bag to the desired weight. Re-clamp lead.
12. Remove transfer spike from dextrose bag and discard and aseptically spike the sterile water bag. Tare the scale. Release clamp on the sterile water lead and by gravity drain into the PN bag to the desired weight. Re-clamp lead.
13. Disconnect lead set and seal PN bag.
14. Mix the solution thoroughly until no dextrose strands can be seen in the solution.
15. Draw up additives by syringe and add in the following order ensuring a thorough mixing after each additive is added to the solution.
    A. Calcium gluconate
    B. Sodium chloride
    C. Magnesium sulphate
    D. Iron
    E. Micro plus 6 pediatric
    F. Potassium phosphate
16. vitamin B_{12} and MVI pediatric should be added to the solution just prior to administration.

ALUMINUM INJECTION PREPARATION

The aluminum injections for the Low and High aluminum (Al) piglets were in the form of aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$) (Fisher Scientific, Mississauga, Ontario, Canada). The aluminum was mixed in 250 mL bags (Abbott Laboratories, Montreal, Quebec, Canada) of normal saline (Abbott Laboratories, Montreal, Quebec, Canada). The final concentrations of the solution were 20 $\mu g \cdot mL^{-1}$ for the Low Al group and 1500 $\mu g \cdot mL^{-1}$ for the High Al group.

The procedure:

1. Using aseptic techniques, dissolve 0.0447 g (for 20 $\mu g \cdot mL^{-1}$) or 3.3542 g (for 1500 $\mu g \cdot mL^{-1}$) $AlCl_3 \cdot 6H_2O$ in approximately 10 mL of sterile water (Baxter Coroperation, Mississauga, Ontario, Canada).
2. Transfer and filter the solution into an empty intravenous (iv.) bag (Abbott Laboratories, Montreal, Quebec, Canada) and then mix with normal saline (Abbott Laboratories, Montreal, Quebec, Canada) to 250 mL.
3. Label the bag and store in the fridge.

Note: The same lot numbers of aluminum chloride hexahydrate, saline, and sterile water were used for each bag prepared.

The calculation notes:

1. To manufacture a concentration of 20 $\mu g \cdot mL^{-1}$ (or 1500 $\mu g \cdot mL^{-1}$) of Al^{3+} using $AlCl_3 \cdot 6H_2O$
2. $AlCl_3 \cdot 6H_2O$ has a molecular weight of 241.5.
3. Every 241.5 µg of $AlCl_3 \cdot 6H_2O$ contains 27 µg Al^{3+}.
4. X µg $AlCl_3 \cdot 6H_2O$ must be added to each mL to form 20 $\mu g \cdot mL^{-1}$ (or 1500 $\mu g \cdot mL^{-1}$) Al^{3+} solution.
   20 $\mu g \cdot mL^{-1}$ (or 1500 $\mu g \cdot mL^{-1}$) / X $\mu g \cdot mL^{-1}$ = 27 µg / 241.5 µg
   X = 178.9$\mu g \cdot mL^{-1}$ (for 20 $\mu g \cdot mL^{-1}$) or X = 13416.7 $\mu g \cdot mL^{-1}$ (for 1500 $\mu g \cdot mL^{-1}$)
5. For a 250 mL bag
   20 $\mu g \cdot mL^{-1}$: 178.9 $\mu g \cdot mL^{-1}$ x 250 mL = 44725 µg = 0.0447 g
   1500 $\mu g \cdot mL^{-1}$: 13416.7 $\mu g \cdot mL^{-1}$ x 250 mL = 3354175 µg = 3.3542 g
6. 0.0447 g $AlCl_3 \cdot 6H_2O$ was transferred into a 250 mL bag to result in a concentration of 20 $\mu g \cdot mL^{-1}$ Al^{3+} solution.
   3542 g $AlCl_3 \cdot 6H_2O$ was transferred into a 250 mL bag to result in a concentration of 1500 $\mu g \cdot mL^{-1}$ Al^{3+} solution.

Appendix C

Results from the individual infants

This appendix lists the results of all 16 infants in the prospective study.

Table 6.1 Results from the individual patients in the PNAC group in the infant prospective study

Patient	Gender	Birth weight (g)	Gestation age (weeks)	Duration of PN (days)	Diagnosis	Direct bilirubin (μmol·L^{-1})				Serum aluminum (μg·L^{-1})			
						Day0	Day7	Day14	Day 21	Day0	Day7	Day 14	Day 21
1	male	1510	31	19	Intestinal atresia	14	54	100	68		530	410	290
3	male	1670	32	12	Intestinal atresia	5	7	49		40	370	15	15
4	male	2380	35	12	Gastroschisis	0	15	27	46	15	20	15	15
11	male	2290	36	21	Intestinal atresia	6	18	32	42	40	1050	40	40
15	male	3120	38	16	Meconium peritonitis	16	26	36		75	710	150	

The empty spaces in the table indicated that the infant was discontinued the PN therapy.

Table 6.2 Results from the individual patients in the non-PNAC group in the infant prospective study

Patient	Gender	Birth weight (g)	Gestation age (weeks)	Duration of PN (days)	Diagnosis	Direct bilirubin (μmol·L^{-1})				Serum aluminum (μg·L^{-1})			
						Day0	Day7	Day14	Day 21	Day0	Day7	Day 14	Day 21
2	male	3627	40	7	Intestinal atresia	11	0			15	260		
5	female	3050	36	10	Gastroschisis	5	14	13	21	450	40	150	15
6	female	3030	38	15	Gastroschisis	0	10	7	7	110	640	240	52
7	female	2720	37	14	Gastroschisis	0	0	0		40	970	91	
8	female	2540	38	9	Intestinal atresia	5	7			260	15		
9	female	1700	33	10	Intestinal atresia	5	9	18	13	440	1420	460	15
10	female	2470	35	25	Gastroschisis	5	6	0	11	20	200	99	15
12	male	900	26	35	Intestinal atresia	10	8	10	12	40	1050	40	40
13	male	3300	42	13	Intestinal atresia	7	0	0		40	90	15	
14	male	3150	39	11	Gastroschisis	6	11			40	520		
16	male	1360	31	30	Gastroschisis	5	7	28	27	15	40	80	80

The empty spaces in the table indicated that the infant was discontinued the PN therapy.

Appendix D

Quality control of aluminum analysis

The concentration of aluminum was determined at Saskatchewan Research Council (SRC), Saskatoon by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). The ICP-AES contains a plasma, a spectrometer and a detector. The plasma is a stream of Argon gas that is ionized by an applied radio frequency field and where the specific elements emit light. The spectrometer can separate the characteristic wavelengths, and the detector can measure the emission spectra. The detectable limit is 30 $\mu g \cdot L^{-1}$. A variety of quality control techniques are used to ensure the validity of routine analytical results. With each technique, results must be within specified limits or corrective action is taken. Quality control was performed on:

1. Control standards of aluminum (prepared by SRC) and standard reference of aluminum (prepared by the author) are analyzed with each batch of samples.
2. One of every ten samples, which was analyzed in duplicate. The limit of quantification is within 10%.
3. Control standards of aluminum (prepared by SRC) were analyzed after every five samples to check the calibration.
4. In each batch of samples, sample matrix effects are checked by spiking one sample with a known amount of aluminum standard (prepared by SRC) and measuring the recovery of the aluminum in the sample. The rate of recovery is within the range from 95% to 105%.

Appendix E

Techniques in the piglet model development

This appendix describes some techniques used to resolve the problems we encounter in the piglet model. A total of 36 piglets were studied, including the pilot study (Table 6.1). Lame symptom, catheter problem, and incision swollen in the piglets were the three major problems we met in the study.

The lame symptom was developed in 15 piglets. The diagnosis of arthritis in these piglets was made from the necropsy reports from piglet 22 and piglet 23. To prevent the arthritis and incision swollen, several techniques were applied:

1. Antibiotics (Excenel, 3mg·kg^{-1}·day, intramuscular) (Pharmacia Animal Health, Missasaga, Ontario) was used daily from the reception date to the end of the study.
2. Op-site Incise Drape (a plastic drape adhere to the patient over the area of the body where the operation will take place) (Smith&Nephew, St. Laurent, Quebec, Canada) was used on the surgery skin area during the operation to prevent infection.
3. Bactroban oinment (Glaxo Smithkline, Missasaga, Ontario, Canada) was applied to the incisions daily until the incision was healed without complication.

It was a challenge to keep the catheter in the growing piglets for three weeks. Catheter problems including the obstruction of catheter and out of vein (because of piglets' scratching) were seen in 14 piglets. To reduce the catheter problem, the following techniques were used:

1. The patency of catheter was maintained by flushing saline twice a day.
2. The use of swabable IV connector (CL2000 connector, ICU Medical Inc., San Clemente, USA) on the end of the catheter reduced the chances of plugged catheter.
3. A special jacket (fix and protect the catheter on the piglet's back) was developed to protect the catheter.
4. Frequent visits (every 2 to 3 hours during the day) to check on the piglets were crucial to finding the problem early.

Table 6.3 Several problems in the piglets during the experiment

Animal	Valid	Lame	Incision	Catheter	Comments
Control					
11	√			X[5]	
12			X	X	Euthanized on day 7
14	√				
16	√				
21	√	X[3]			
23		X		X	Euthanized on day 8 and sent for
25	√	X[3]		X[5]	
Subtotal	**n=5**	**n=3**	**n=1**	**n=4**	
Low Al					
1[2]				X	
2[2]					
3[2]				X	
4[2]					
5[2]		X	X	X	
6[2]				X	
7	√	X[3]		X[5]	
8	√	X[3]		X[5]	
9	√				
10	√				
13		X			Euthanized on day 14
17		X			Euthanized on day 7
20	√				
22		X		X	Euthanized on day 14 and sent for
24	√				
26	√	X[3]			
Subtotal	**n=7**	**n=7**	**n=1**	**n=7**	
High Al					
15		X		X	Euthanized on day 17
18	√	X[3]			
19	√				
27	√				
28	√				
29	√			X[5]	
30	√	X[3]			
Subtotal	**n=6**	**n=3**	**n=0**	**n=2**	
PN					
31	√	X[3]			Euthanized on day 14
32	√		X[4]	X[5]	Euthanized on day 14
33	√				
34	√				
35	√				
36	√				
Subtotal	**n=6**	**n=1**	**n=1**	**n=1**	
Total	**n=24**	**n=14**	**n=3**	**n=14**	

1. The valid data means the data used in the piglet study in the experiment part of the thesis.
2. These piglets were in the pilot study (numbers1-6).
3. Minor lame symptom and rescovered one or two days later.
4. Minor incision swollen and resolved one day after.
5. Minor catheter obstruction, used saline flushed and resolved

Appendix F

Results from the individual piglets

This appendix lists the results of total bilirubin, serum aluminum concentration, liver aluminum concentration, urine aluminum concentration, and bile aluminum concentration from all 24 individual piglets in the experimental study. The concentration of aluminum was determined at Saskatchewan Research Council (SRC), Saskatoon by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). For the piglets (number 7, 8, 9, 10, 11, 14, 16, 18, 19, 20, 21, 24), the detectable limit of aluminum is 30 $\mu g \cdot L^{-1}$ (method: ICP-AES, instrument model: TJA-ICAP-61E). For the piglets (number 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36), the detectable limit of aluminum is 2.5 $\mu g \cdot L^{-1}$ (method: ICP-AES, instrument model: G3152A). For statistical purposes, when the aluminum level was below the detectable limit, half of the value was used in the table.

Table 6.4 Results from the individual piglets in the control group in the experimental study

Piglet number	Total bilirubin (μmol·L^{-1})				Serum aluminum (μg·L^{-1})				Liver aluminum (μg·g^{-1})	Urine aluminum (μg·L^{-1})	Bile aluminum (μg·L^{-1})
	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21			
11	2	1	1	1	45	15[1]	15[1]	15[1]	0.08	15[1]	15[1]
14	1	2	2	2	90	15[1]	15[1]	90	0.14	15[1]	15[1]
16	1	3	3	1	63	15[1]	15[1]	44	0.01	30	15[1]
21	0	1	3	3	15[1]	15[1]	100	15	0.01	15[1]	15[1]
25	1	2	2	1	15	39	22	24	0.02	20	20

1. The aluminum concentration < 30 μg·L^{-1}, half of 30 μg·L^{-1} (15 μg·L^{-1}) was used in the table.

Table 6.5 Results from the individual piglets in the Low Al group in the experimental study

Piglet number	Total bilirubin (μmol·L^{-1})				Serum aluminum (μg·L^{-1})				Liver aluminum (μg·g^{-1})	Urine aluminum (μg·L^{-1})	Bile aluminum (μg·L^{-1})
	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21			
7	2	9	2	2	15[1]	15[1]	15[1]	15[1]	1.40	15[1]	65
8	2	3	6	8	50	15[1]	15[1]	15[1]	0.48	15[1]	150
9	2	2	2	2	160	780	260	90	0.33	15[1]	15[1]
10	2	2	2	4	15[1]	110	150	60	0.27	190	60
20	0	1	2	1	40	15[1]	80	50	0.52	15[1]	15[1]
24	1	2	3	2	60	630	230	230	1.48	110	80
26	3	3	2	2	30	110	130	90	1.60	230	60

1. The aluminum concentration < 30 μg·L^{-1}, half of 30 μg·L^{-1} (15 μg·L^{-1}) was used in the table.

Table 6.6 Results from the individual piglets in the High Al group in the experimental study

Piglet number	Total bilirubin (μmol·L^{-1})				Serum aluminum (μg·L^{-1})				Liver aluminum (μg·g^{-1})	Urine aluminum (μg·L^{-1})	Bile aluminum[2] (μg·L^{-1})
	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21			
18	1	3	6	2	15[1]	480	270	270	28	3840	15[1]
19	3	2	8	2	15[1]	820	320	600	17	1100	90
27	1	8	5	2	39	1230	578	630	100	477	130
28	1	14	5	10	50	1300	1320	300	94	1520	250
29	1	5	7	3	30	1800	2050	2420	100	1460	960
30	1	3	10	15	10	1490	1680	1060	89	729	

1. The aluminum concentration < 30 μg·L^{-1}, half of 30 μg·L^{-1} (15 μg·L^{-1}) was used in the table.
2. There was no bile that could be obtained from piglet 30 at the end of the study.

Table 6.7 Results from the individual piglets in the PN group in the experimental study

Piglet number	Total bilirubin (μmol·L^{-1})				Serum aluminum (μg·L^{-1})				Liver aluminum (μg·g^{-1})	Urine aluminum (μg·L^{-1})	Bile aluminum[3] (μg·L^{-1})
	Day 0	Day 7	Day 14	Day 21[2]	Day 0	Day 7	Day 14	Day 21[2]			
31	2	3	47		9	30	28		0.78	126	5
32	1	7	21		18	27	30		0.52	269	20
33	3	48	122	177	14	40	54	70	1.50	144	
34	2	14	74	131	16	114	57	30	1.07	232	
35	2	6	26	86	11	33	66	24	0.73	646	
36	2	3	87	44	16	25	35	24	0.80	136	25

1. Piglet 31 and piglet 32 were on PN therapy for 14 days.
2. There were no bile for piglet 33, 34, and 35 at the end of the study.